Influence of Nutrient Availability on Soil Respiration and Microbial Activity in a Tree-Grass Ecosystem

Dissertation

To Fulfill the

Requirements for the Degree of

„Doctor of Philosophy" (PhD)

Submitted to the Council of the Faculty

of Biological Science

of the Friedrich Schiller University Jena

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Dissertation, Friedrich-Schiller-Universität Jena, 2020

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Kendalynn Ann Morris

Acknowledgements

I would like to thank my supervisors, Mirco Migliavacca and Marion Schrumpf, for their incredible support in letting me pursue my degree on my own terms. It has been a life-changing opportunity to complete my doctoral research at the MPI-BGC. A part of me will forever be just a little Deutsch now. My advisory committee also has my gratitude for being a powerful motivating force throughout my four years. An especially big thanks goes to Gerardo Moreno Marcos, for sharing his passion and depth-of-understanding for the dehesa ecosystem. It was truly a pleasure to study this quintessential part of Extremadura, in large part due to support from him and his incredible group.

Richard Nair and Tiana Hammer deserve distinctive recognition as my primary companions in the field. I always look back on my fieldwork with fondness; we worked really hard, but we had good time doing it.

A very warm thanks to Jessica Heublein and Theresa Klötzing, for their welcoming attitude when I first arrived. A special thank you to Lucia Eder as my primary guide in navigating German culture, translating forms, and making sense out of whatever came in the mail. She also graciously prepared the German translation of the summary contained herein. Thanks is also extended to the many dedicated experts in the Freiland, ROMA, Stable Isotope, and ^{14}C-analytical service groups for making experimental research at the institute possible.

I thank also my family, who with great restraint, only very occasionally made me feel guilty for living so far away. You always believed in me.

Finally, thank you to Andrew Durso, my soon to be husband. You knew from the beginning how hard it would be, but you supported me anyway, and uprooted your entire life so we could share this incredible adventure. Thank you.

Chapter 1

1.1 Motivation

Climate change has brought unprecedented awareness of global carbon (C) cycling to both the public and academic sphere. While there is no doubt that the root cause of increased atmospheric carbon dioxide (CO_2) is anthropogenic emissions, research on the topic has exposed many aspects of C cycling that we do not fully understand. One major gap is understanding the extent to which availability of other major nutrients (namely nitrogen (N) and phosphorus (P)) controls C cycling (Zaehle et al. 2010, Peñuelas et al. 2012, Stiles et al. 2017). Much of the research on this topic is driven by another anthropogenic forcing, atmospheric N deposition. Because N is typically the dominant limiting nutrient for many terrestrial ecosystems (LeBauer and Treseder 2008), the increase in deposition related to anthropogenic activities, such as fossil fuel combustion, could lead to increased C uptake with potential increases in C storage due to release from N limitation (Magnani et al. 2007). However, if ecosystems already have sufficient N available to meet demands of primary producers, N deposition will shift the system towards P limitation because anthropogenic input of P does not keep pace (Peñuelas et al. 2012). The conditions under which N deposition would lead to deviations from optimal ecosystem N:P stoichiometry are important to understanding global C cycling, not only in terms of a response of plants, but also because of potential impacts on soil microbes. There is strong evidence that addition of N decreases the enzyme activity and biomass of soil microbes (Treseder 2008, Ramirez et al. 2012), but the relationship between P addition and microbial activity is less clear (Ramirez et al. 2012, Poeplau et al. 2016, Mooshammer et al. 2017). Furthermore, the response of microbes to addition of N and P together often differs from their response to addition of one alone (Poeplau et al. 2016, Mooshammer et al. 2017). If increased N and P availability or changes in N:P stoichiometry of soil increases microbial C processing, this could overwhelm any gains in C uptake achieved by vegetation. This potential compensatory effect of microbes is difficult to detect however, because a primary method that we have for measuring *in situ* rates of decomposition is to measure soil CO_2 efflux, which by its very nature is a mixture of heterotrophic and autotrophic respiration (Hanson et al. 2000). Nutrient controls on soil C processes are generally poorly understood in natural systems and are inherently difficult to study due to high spatial heterogeneity and difficulty of finding sites with comparable soil and climate properties, but different nutrient availabilities (Carter and Gregorich 2008). To alleviate this problem, nutrient addition experiments can be performed in ecosystems that naturally contain soils with contrasting properties. Oak-savannas are mixed tree-grass ecosystems where, due to *in situ* decomposition of tree detritus, there are large patches of soil with higher

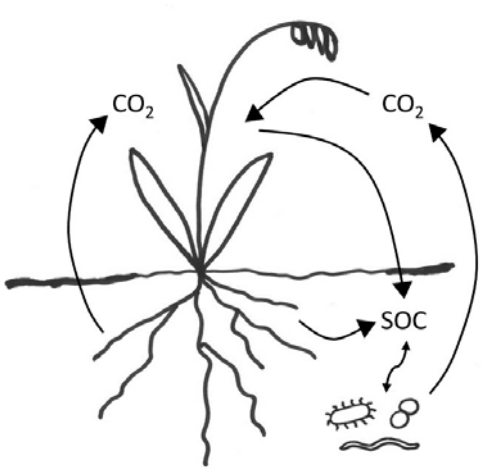

Figure 1. Simplified terrestrial carbon cycle, showing the processes considered in this text. SOC stands for soil organic carbon.

concentrations of soil organic C (SOC) than that found in the surrounding open grassland (Gallardo 2003, Moreno et al. 2007). These systems are seasonally dry, making them important to understanding the link between nutrient cycling and water availability. The goal of this dissertation is to quantify how N and P availability influence soil C cycling in an oak-savanna using multiple approaches.

1.2 The Carbon Cycle

Our planet is maintained within the narrow bounds of livable conditions by numerous interacting processes which constantly redistribute energy and renew materials via various biogeochemical cycles. Life on earth is carbon (C) based, a basic fact which until the industrial revolution was of primarily academic import. Since that time however the release of excess carbon dioxide (CO_2) into the atmosphere, a by-product of using fossil fuels for energy, has fundamentally changed how C is distributed throughout the critical zone. Often called the greatest challenge of our time (Karl and Trenberth 2003, Dow and Downing 2016), this on-going perturbation to the global C cycle is accompanied by additional anthropogenic changes, such as increased nutrient availability and changes in climate, that contest our understanding of how C cycling works (Schlesinger 2009, Peñuelas et al. 2012). Such knowledge gaps urgently need to be filled because this understanding is essential to making informed decisions regarding climate change mitigation.

The C that makes up living things has two dominant fates; it can return to the atmosphere as CO_2 in a process known as respiration, or it can remain locked in biomass until its host organism dies (Figure 1). Luckily, dead biomass does not last long, and is soon processed by decomposers, becoming again biomass (and possibly CO_2). Alternatively, if the dead biomass is on land, it becomes part of the soil organic C (SOC) pool. SOC consists of any organic compound of any size contained within the soil, including but not limited to dead roots, plant litter, and microbial biomass. SOC acts as a food source for soil microbes, making this pool, and the soil itself, a critical junction in C cycling.

1.2.1. The role of soil in the carbon cycle

Soils are the biggest actors in terrestrial C cycling because they store vast quantities of C as SOC globally (1,400 Pg, Post et. al., 1982). This C originates from plants, and exactly how it accumulates remains

unknown (Lehmann and Kleber 2015), but it is the result of long-term accumulation of a very small net increment. In this sense, the photosynthetic fixation of CO_2 by primary producers in the ecosystem acts as the input, and the dominant control on the total amount of C that can be stored in soils, and respiration of decomposers, especially prokaryotes and fungi, as a result of their metabolism are the outputs, blowing away much of what plants have fixed. Soil respiration is the second most important flux in the terrestrial C cycle (Bond-Lamberty et al. 2018). It has recently emerged that the anabolism of C by soil microbes is also important in SOC stocks (Liang et al. 2017), as any C which is not immediately respired has an increased chance of persisting in the soil system for long periods of time as part of microbial biomass. Various aspects of plant C fixation and physical allocation as well as microbial C metabolism are affected by environmental conditions and species-specific traits, making the relationship between the major input and output of the SOC budget as variable as ecosystems themselves.

1.2.2 Soil respiration

As described above, respiration from living organisms is an important component in the C cycle. Countless billions of microorganisms make their home in the soil and respire there. The CO_2 they produce is joined with another source of CO_2, that which is respired from plant roots. This second source of soil respiration is often surprising to those not familiar with soils, because we often ignore the metabolic demands of plants, instead focusing on the fact that they take-in CO_2 for photosynthesis (Figure 1). Naturally however, much of the sugar they produce is what they use to grow and subsequently, respire. Soil respiration is one of the most readily measured aspects of soil C cycling, because this respired CO_2 passively exchanges with the atmosphere, a process more accurately called soil CO_2 efflux. The challenge lies in that it is often not enough to know the flux, rather what we need to know is the proportion of CO_2 that came from plant roots and soil microbes, as well as the environmental conditions that are controlling these contributions. By partitioning soil respiration between these two sources we can better understand if the SOC pool is likely shrinking or growing (Sapronov and Kuzyakov 2007).

1.3 N and P stoichiometry

Organisms contain much more than just C. By dry weight, N and P are the second and third most abundant elements in biomass. Stoichiometry, as it relates to the elemental composition of living things, refers to their abundance in biomass and its components, typically relative to C, but sometimes relative to each other. The majority of biochemical substances have a fixed chemical composition that is then reflected in their stoichiometry, and this idea can be scaled to larger tissues and even whole organisms.

For example, DNA has a C:N of 2-5:1, while the green leaf of a plant would have a C:N of 20-40:1. These same substances would have a C:P of 9-10:1 and 200-400:1 (Elser et al. 1996). The ratios vary widely because N and P play very different roles in living cells. Both are essential in nucleic acids, but N is more important in amino acids, as enzymes and proteins, while P is more important for structural components and energy. Their elemental cycling is also different, with N having many different redox states which lends it to extensive metabolic processing by prokaryotes which P lacks (e.g., nitrification).

1.3.1 Linking C, N, and P Cycling

Because C, N, and P are all part of organisms, they are also all found in SOM and it is through the decomposition of SOM that most N and P is released back into the soil in a bio-available form (Figure 2). This process is called mineralization because it describes the transformation of material from an organic to an inorganic or mineral form. Without the mineralization of nutrients like N and P by microbes, the soil would become a barren wasteland and life on earth would cease to exist. However, microbes are not performing this ecosystem service for their own benefit, rather as a by-product of using SOM to fuel their growth and metabolism, meaning that as microbes decompose SOM, they release excess N and P as waste products. This comes about because heterotrophic microbes need organic substances not just for building biomass, but also for their energy. Typically, more than 50% of the C taken-up by a microbe will be used to produce energy for the cell (Manzoni et al. 2012, Sinsabaugh et al. 2013, Hagerty et al. 2018). The remainder will be incorporated into biomass, along with as much N and P (and other nutrients) as are needed to maintain the microbe's stoichiometry. Often, this remainder contains more N or P than the organism needs, resulting in mineralization. If not, then additional sources of N or P, both organic and inorganic are taken in. This is called immobilization because the nutrients become bound or immobilized within the microbial biomass. Therefore, the amount of C that microbes put into new biomass out of all the C that they take-in, their C-use efficiency (CUE), is a primary determinant of microbial demand for N and P and the rate at which these nutrients

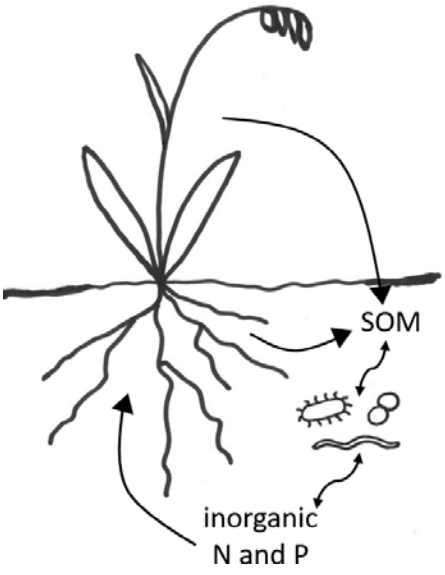

Figure 2. Simplified terrestrial nitrogen and phosphorus cycles, showing the processes considered in this text. SOM stands for soil organic matter, which contains both N and P.

are cycled. It is also a critical parameter in global C cycling because it defines the microbial contribution to soil respiration.

1.3.2. Effects of N and P on soil carbon cycling

Because the behavior of plants and soil microbes varies depending on environmental conditions, it has been proposed that the soil C sink can be enhanced through proper ecosystem management to help mitigate CO_2 emissions (Lal 2004). Whether or not this is a viable strategy is still an open question, and dependent on resolving how nutrient addition influences SOC stocks. The addition of mineral N (fertilization) can lead to decreases in the respiration of SOC because of decreased plant and microbial demand for N (Janssens et al. 2010, Ramirez et al. 2012, Riggs et al. 2015). If the decrease in respiration is large enough, and input is maintained or increased with fertilization, SOC stocks will increase. However, it is also possible that N addition will have the opposite effect, leading to increased respiration if soil microbes become C limited (Hobbie and Hobbie 2013, Leff et al. 2015). Naturally, these two scenarios are simplifications because soil respiration is also influenced by climate (Davidson et al. 1998, Davidson and Janssens 2006), soil texture (Wang et al. 2003, Cable et al. 2008), vegetation (Lai et al. 2014), microbial communities (Monson et al. 2006), and availability of other essential nutrients such as P (Teklay et al., 2006), and the scale at which the system is examined influences which determines which drivers are the most important (Reichstein and Beer 2008).

Because of the importance of fertilizer to increasing agricultural yields, the majority of research investigating nutrient-addition effects on SOC focuses specifically on N addition in agricultural soils. In general, addition of N increases C storage as SOC (Alvarez 2005), although total C sequestered does not always compensate for 'hidden' C costs incurred during land management (Lal 2004, Alvarez 2005). Beyond agricultural studies, Fog (1988) reviewed the influence of N addition on the decomposition of organic matter and found over-whelming support for a decrease in microbial respiration. Recent work on natural grasslands (Riggs et al. 2015) generally agrees with these findings, although the reduction in respiration was only seen in certain soil C pools. In each of these instances the mechanisms by which decomposition is slowed or respiration is reduced are poorly understood. Janssens *et al* (2010) propose two possibilities; 1) reduction in belowground allocation by plants and 2) shifts in the microbial community.

In the first scenario, plant root growth and exudation is decreased by N addition because of the increased ease with which N can be taken up (Phillips and Fahey 2007). This leads directly to less absolute root respiration and indirectly to less microbial respiration due to the decrease in available C for

metabolizing. In the second scenario, increased availability of N favors fast-growing microbes that selectively metabolize more labile substrates, these then prevent or inhibit decomposers that utilize the larger, more recalcitrant C pool (Ramirez et al. 2012). In order to test these hypotheses, experiments must be conducted that combine N addition with comprehensive studies of soil C dynamics and microbial community structure.

Because of the great quantity of N in biomass, it is often the limiting nutrient in ecosystems (LeBauer and Treseder 2008). This fact, in combination with widespread atmospheric deposition (Reay et al. 2008) has led to a focus in scientific studies on N addition effects. However, given long-term N deposition ecosystems may be shifting towards P limitation (Vitousek et al. 2010, Peñuelas et al. 2012). One way of conceptualizing N vs P limitation is to utilize N:P ratios. Sites with low N:P would be N limited, but P rich, while sites with relatively high N:P might be P limited. Relatively little is known about how P addition alone affects SOC and the few studies that have been conducted have mixed results (Torn et al. 2005, He and Dijkstra 2015, Huang et al. 2018). The uptake of N and P by soil microbes is regulated by different mechanisms, so depending on specific environmental conditions addition of them together can lead to differential effects to their addition alone (Olander and Vitousek 2000, Chubukov et al. 2014). Therefore, it is essential to study the synergistic effects of N and P addition alongside their individual effects to enhance our understanding of how changes in nutrient availability will influence soil C cycling.

1.4 Tree-grass ecosystems

Tree-grass ecosystems are found in many parts of the world and are simply defined as consisting of a mixture of these two vegetation cover types. The majority of tree-grass ecosystems, also known as savannas, are found in semi-arid environments which makes them especially sensitive to the effects of climate change (Mistry and Beradi 2000, Hanan and Lehmann 2010). Additionally, these semi-arid regions play a key role in global C budgets, contributing disproportionally to the interannual variability of C uptake (Poulter et al. 2014, Ahlström et al. 2015). These are dynamic ecosystems, often exhibiting strong seasonality where factors limiting growth shift throughout the year from temperature, to nutrients, to water (Mistry and Beradi 2000, Vitousek et al. 2010, Nair et al. 2019).

Dehesas are semi-agricultural tree-grass ecosystems dominated by oak-savanna. They cover between 3.5 and 4 million hectares of the Iberian Peninsula and are important for cultural, economic, and ecological reasons (Joffre et al. 1999, García and Mata 2000, Olea and San Miguel-Ayanz 2006). Historically, dehesas were established on sites that were unsuitable for traditional agriculture and since establishment have maintained their distinct vegetative cover for centuries (Stevenson and Harrison 1992,

Olea and San Miguel-Ayanz 2006, Ferraz-de-Oliveira et al. 2016). Like all oak-savanna systems, dehesas are defined by their patchiness, with an open canopy of mostly holm oak (*Quercus ilex* L.) and an understory of grasses and forbs. In addition to this spatial heterogeneity, the Mediterranean climate has a distinctly seasonal distribution of moisture.

The soil samples used, as well as the field measurements made, for the research described in this text originated from a specific dehesa near the village of Majadas in Extremadura, Spain. The Majadas dehesa is 258 m above sea level and receives 650 mm of precipitation, almost exclusively in the winter months. It has a tree density of roughly 20 trees ha^{-1} and is grazed from early December to late June by cattle at an intensity of < 0.3 livestock units ha^{-1} y^{-1}. The soil is an Abruptic Luvisol with 79% sand, 20% silt, and 1% clay in the surface horizon under tree canopies, and 74% sand, 20% silt, and 6% clay in the surface horizon in open areas. Soils that developed under oak canopies at Majadas have on average 66% more organic matter in the surface horizon and a moderated microclimate relative to open spaces, which is a typical characteristic of dehesas (Gallardo 2003, Howlett et al. 2011).

1.5 Objectives, structure, and attributions

In order to better understand how N and P availability influence soil C cycling, in 2015 a large-scale N and P manipulation experiment (MANIP) was established in the Majadas dehesa. The use of the dehesa ecosystem allows for the influence of vegetation, soil moisture, and nutrient addition to be studied while climate, soil texture, and initial biological communities are kept constant. As mentioned above, although the vast majority of research has found that nutrient addition decreases soil respiration, some previous work has found that low fertility sites actually had increased soil respiration with fertilization (Torn et al. 2005). Because of the close proximity of soils with high and low SOC in dehesas, we have the potential to see such differential results and determine the mechanism(s) underlying them.

The overall research questions of this thesis are focused on the effect of N and P availability on soil respiration, the partitioning of N within ecosystem components, and microorganism activity, as well as how each of these differs within the two distinct habitats of the dehesa. It is organized into three studies, presented herein as three data-chapters, each dealing with one of these different aspects. The final chapter of this thesis is a general discussion of the results including an overall conclusion and future outlook.

1) How does soil respiration respond to changes in N and P availability?
   a) Title: *Habitat and soil N:P stoichiometry control soil respiration in a Mediterranean oak-savanna*

b) For this data-chapter I measured soil respiration in sampling campaigns from March 2017 to May 2018 at the MANIP experimental site. I used data from manual-measurements and automated-chambers to model shifts in basal respiration and the temperature sensitivity of soil respiration in response to changes in available N:P.

c) Contributions: Kendalynn Morris designed, collected, and analyzed the manual-measurement based experiment. Thomas Wutzler gap-filled data and conducted the temperature and sensitivity analysis. Tiana Hammer collected respiration data and assisted with laboratory work. Marco Poehlmann installed and maintained the automated chambers. Marion Schrumpf, Mirco Migliavacca, and Gerardo Marcos designed the original large-scale fertilization experiment and contributed to the design of the sub-experiments reported here.

2) How does the availability of P influence the fate of added N?

a) Title: *Fate of N additions in a multiple resource limited Mediterranean oak-savanna*

b) The second data-chapter reports on a field-based stable isotope tracer experiment tracking ecosystem allocation of added N through surface soil, plants, and soil microbes with and without P addition in two habitat types. To do this I established a small-scale duplicate of the MANIP experiment in 2017 and added ^{15}N to determine the fate of N over the course of one year.

c) Contributions: K. Morris designed and analyzed the experiment, Richard Nair and K. Morris collected the data and contributed to installation, G. Moreno helped install and worked on the initial experimental development, M. Schrumpf and M. Migliavacca helped to develop the experimental design. All authors worked on data interpretation and manuscript preparation.

3) How does N and P addition influence microbial activity?

a) Title: *Microbial carbon-use efficiency is not limited by the availability of nitrogen and phosphorus*

b) In order to isolate the microbial response to shifts in N:P availability, for my third data-chapter I conducted a laboratory study. To quantify microbial activity, I measured microbial carbon-use efficiency and a suite of related variables such enzyme activity and the isotopic signature of respired C, on samples with short-term and long-term histories of fertilization.

c) Contributions: K. Morris developed the original concept of the experiment, generated the majority of the data, analyzed the data, and wrote the manuscript. Andreas Richter facilitated the collection of CUE data and its interpretation. Kirsten Küsel facilitated the collection of taxonomic

data. All authors contributed to the experimental design, data interpretation, and manuscript prep.

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Chapter 2

Habitat and soil N:P stoichiometry control soil respiration in a Mediterranean oak-savanna

Abstract

Soil respiration is one of the major fluxes in the global carbon (C) cycle, linking plant uptake and microbial decomposition on land. Plants and soil microbes, like all living organisms, require nutrients as part of their growth and maintenance. Worldwide ecosystems are experiencing changes to their C cycling due to increased anthropogenic nitrogen (N) and phosphorus (P) inputs. Extensive study shows that forest ecosystems tend to have decreased soil respiration in response to N addition, while grasslands generally have the opposite response, but the mechanisms behind this difference are still poorly understood and very few studies include effects of P addition. To expand our understanding of how ecosystem properties modify the effect of nutrient addition on soil respiration, we measured soil respiration and derived its temperature sensitivity (Ea) and the basal respiration rate at a reference temperature of 10˚C (Rb) in the two main habitats of an oak-savanna, under tree canopies and in open grassland areas. These habitats differ in their soil organic matter (SOM) content, which is almost 3 times greater under canopies, as well as the presence of tree roots. We established combinations of automated soil respiration chambers and soil sampling points in control and N addition plots as well as subsets of these sampling types in plots fertilized with both N and P (+N+P) and P alone. We found that habitat played a major role in the over-all behavior of soil respiration as well as its response to nutrient addition, with open grassland areas having significantly increased respiration and increased Ea of respiration with N addition, and under canopy soil having a trend towards the opposite response. The response to N alone was much stronger than the response to +N+P or P alone, meaning that this ecosystem is sensitive to increases in the N:P ratio of available nutrients. Our results indicate that SOM content is an important predictor of how soil respiration will respond to nutrient addition.

Introduction

Soil respiration, the flux of carbon dioxide (CO_2) which leaves the soil surface, is the sum of microbial respiration and respiration from plant roots. It plays a key role in linking autotrophic CO_2 fixation and heterotrophic CO_2 release in the largest terrestrial carbon (C) reservoir, the soil (Schlesinger and Andrews 2000). How much fixed C leaves the soil via heterotrophic espiration is an important modifier determining annual ecosystem C storage, making this C flux a critical component of global C cycling (Bond-

Lamberty et al. 2018) accounting for about 90 Pg C yr^{-1} (Bond-Lamberty and Thomson 2010, Hashimoto et al. 2015). Soil respiration rates are sensitive to the addition of mineral nutrients, especially nitrogen (N), but the response is highly variable (Janssens et al. 2010, Riggs et al. 2015, Manzoni et al. 2018). An extensive body of work originating in the early 20^{th} century shows that while many soils increase their respiration rates after addition of N fertilizer, others have the opposite response (Van Suchtelen 1910, Potter and Snyder 1916, Janssens et al. 2010, Peng et al. 2011).

What is interesting about this simple experiment is that the results are so easily explained in either direction. Decreased soil respiration could be due to decreased abundance of plant roots (suddenly not needed due to greater nutrient availability), leading to less autotrophic respiration (Magnani et al. 2007, Janssens et al. 2010). Simultaneously, fewer plant roots can led to less rhizosphere-associated microbial respiration (Magnani et al. 2007, Janssens et al. 2010). Alternatively, increased soil respiration could be due to increased plant growth, increasing the autotrophic contribution, and increased rhizosphere respiration from heterotrophs (Janssens et al. 2010, Riggs et al. 2015). Effects also differ if nutrients other than N, such as phosphorus (P), are added (Kang et al. 2016, Ren et al. 2016, Wang et al. 2017). N and P together might switch the direction of change (Ren et al. 2016) or be consistent with addition of N alone (Kang et al. 2016, Wang et al. 2017). Studies looking at the effect of P are less common (Vitousek et al. 2010, Peñuelas et al. 2012, Ulm et al. 2017), and have not been able to satisfactorily explain the inconsistencies between effects of N alone vs N and P on soil respiration. Greater understanding of this phenomenon is urgently needed because availability of N and P is increasing in many ecosystems worldwide, but rarely at equal rates (Peñuelas et al. 2012).

Temperature sensitivity (Ea) of decomposition is another factor which needs to be taken into consideration regarding soil respiration responses to nutrient addition. The enzyme-catalyzed reactions that underlie such a generalized term as 'respiration' must, as any chemical reaction, have an activation energy which is influenced by the temperature under which the reaction occurs. Under moist (but not water-saturated) conditions, a substantial amount of soil organic matter (SOM) should exhibit positive Ea via increased heterotrophic respiration (Davidson and Janssens 2006). However, if enzyme activities are decreased by N addition (Ramirez et al. 2012), Ea of soil respiration should also decrease because fewer enzymes are participating in the temperature driven response. Alternatively, if the response of soil respiration is driven by plant roots, N addition might result in increased temperature sensitivity (Burton et al. 2002). The interaction of N deposition and increasing temperatures on soil respiration lie at the crossroads of predicting future trends in global C cycling (Davidson and Janssens 2006, Peñuelas et al. 2012), and how this is influenced by availability of P is an open question.

Because site-specific conditions tend to influence all of these factors, it is especially beneficial to study soil respiration and how it is influenced by nutrient addition in tree-grass ecosystems, such as oak-savannas, where two distinct habitats naturally sustain differential soil conditions. Under tree canopies, the in-situ decomposition of tree litter, in combination with seasonally moist and favorable growth conditions support a greater microbial biomass as well as greater quantities of SOM (Gallardo 2003). In contrast, the open grassland areas follow the pattern of an annual grassland, with relatively low SOM accumulation due to limited plant inputs. This creates the advantage of allowing the effect of variable SOM to be tested while maintaining similar soil chemistry, herbaceous communities, and climatic conditions. Additionally, such ecosystems have merit for study in their own right because semiarid ecosystems contribute disproportionately to the interannual variability of the global C budget (Poulter et al. 2014, Ahlström et al. 2015). We took advantage of an established fertilization experiment in a tree-grass ecosystem (El-Madany et al. 2018) to determine the effect of habitat and that of N and P availability on soil respiration, its autotrophic and heterotrophic components, as well as Ea and Rb. We hypothesized the following:

1) Soil underneath tree canopies will show decreased respiration in response to nutrient addition. This is the global trend for the majority of forest soils (Janssens et al. 2010) and under canopy soils should respond similarly due to their forest-like origin.
2) Soil in open grassland will show increased respiration in response to nutrient addition due to being more nutrient-limited soil, which should show strong plant productivity responses to nutrient additions (Peng et al. 2011).
3) Soil underneath tree canopies will show greater Ea of soil respiration than open grassland, because more lignin-based organic matter is present there which should exhibit greater sensitivity (Davidson and Janssens 2006).
4) Ea of soil respiration will increase with nutrient addition in the open grassland because larger quantities of plant-derived C will become available to soil microbes (see H2), facilitating the decomposition of more chemically complex SOM (Conant et al. 2008).

Methods

Study Site

This study was conducted in the oak-savanna outside of Majadas de Tiétar (39°56'25"N 5°46'29"W) in Extremadura, Spain. This oak-savanna has roughly 20 trees ha^{-1} and supports cattle (< 0.3 livestock units ha^{-1} y^{-1}) during the productive period between early December and late June (El-Madany et al. 2018). The soil is an Abruptic Luvisol and the herbaceous layer is an annual-dominated native pasture. The site is at 268 m above sea level and receives approximately 650 mm precipitation annually, most of which falls between winter and early spring. Since 2003 the site has been the location of an eddy covariance tower belonging to the FLUXNET network with the site identifier ES-LMa (El-Madany et al. 2018).

The Majadas oak-savanna is home to a large-scale fertilization experiment designed to study the impact of stoichiometric N:P imbalance (Nair et al. 2019). In winter of 2014 (for P containing fertilizer) and spring of 2015 (for N containing fertilizer), pelleted fertilizers were applied using a GPS equipped tractor to two newly-established eddy-covariance tower footprints in order to create N addition (+N) as well as N and P addition (+N+P) treatments. The +N treatment received calcium ammonium nitrate (100 kg N ha^{-1}N) while the +N+P treatment received a combination of ammonium nitrate (again 100 kg N ha^{-1}N) and triple superphosphate fertilizer (50 kg P ha^{-1}). These treatments were reinforced in the winter of 2015 (10 kg P ha^{-1}) and spring of 2016 (20 kg N ha^{-1}). The main experiment includes the control EC-tower footprint, the +N footprint (high N:P, expected to develop P limitation), and the +N+P footprint (relieved P limitation). Additionally, in the vicinity there is a smaller, and therefore tower-less, P addition plot (+P, low N:P, expected to develop N limitation) which is also included in this study.

Soil Respiration Measurements

Manual Soil Respiration Measurements

To study the effect of N:P stoichiometry and habitat on soil respiration and respiration partitioning, within each of the four treatment plots (control, +N, +N+P, and +P) four sampling points (SP's) were established under canopies and four in the open grassland. SP's were established in North- South running transects each of which centered on an individual oak tree. Under canopy stations were located mid-way between the trunk of a tree and the edge of its canopy and open grassland stations were located at least twice the canopy radius from any trunk. In early December 2016, a soil respiration

partitioning experiment was installed at each SP. Cylinders (30 cm height, 20 cm diameter) constructed of 4, 35, or 400 µm mesh were installed in the ground with minimum disturbance to the soil. This was accomplished by driving a sharpened steel cylinder of the same dimensions into the soil, then extracting this template cylinder using leverage, keeping the soil in place, but creating a negative space that the mesh cylinder was then gently inserted into. On top of each cylinder, a 20 cm diameter respiration collar, designed to fit with the Li-Cor 8100A portable gas analyzer (LI-COR Biosciences, Lincoln, NE, USA), was placed. An additional (fourth) collar was placed on the soil surface with no mesh cylinder underneath. The purpose of the cylinders was to provide a selective barrier to either roots (35 µm) or roots and fungal hyphae (4 µm) as well as a control that allowed these components to enter (400 µm). The fourth collar was included as a disturbance control to measure soil respiration of the fully intact soil system. The bottom lip of the collars was pressed into the soil approximately 1 cm to ensure that no air would flow from underneath would interfere with flux measurements. Vegetation was removed from the surface of all collars and regrowth was kept to a minimum via aggressive weeding and covering the soil surface with a circular cut-out of weed cloth.

Four months after installation, soil respiration measurements began. Measurements were made in semi-seasonal campaigns (Table 1). For each campaign respiration was measured on four consecutive days with similar weather, with all SP's from one nutrient addition plot measured in one day, starting approximately 2 h after sunrise and ending at solar noon. This sampling scheme was necessary due to equipment limitation (only two Li-Cor 8100-A's), the distance between the different plots, and concerns regarding comparability of measurements made later in the day to those made earlier (Vargas et al. 2009, Ruehr et al. 2010). From each collar, four consecutive readings of changes in CO_2 concentration over time were made and the median value was taken. Measurement periods were 90 s in length. Flux values were calculated using the *RespChamberProc* R-package (freely available at https://github.com/bgctw/RespChamberProc) using a 10 s lag time and non-linear fit. Fluxes with an R^2 of the fit below 0.97 were excluded. Starting in the August 2017, soil moisture (8100-204 ML2x, Li-Cor Biosciences, Lincoln, NE, USA) and temperature (6000-09TC, Li-Cor Biosciences, Lincoln, NE, USA) data were collect for each SP concurrent with respiration measurements.

In order to quantify the respective role of plant roots and soil microbes in total soil respiration, the partitioning of soil CO_2 flux was modeled using the following equations:

$$CO_{2\,intact} = CO_{2\,flux\ from\ coreless\ collars}$$

$$CO_{2\,soil} = CO_{2\,roots} + CO_{2\,microbes} + CO_{2\,mycorrhizae}$$

$$CO_{2\ microbes} = CO_{2\ flux\ from\ 4\ \mu m\ collars}$$

$$CO_{2\ roots} = CO_{2\ flux\ from\ 400\ \mu m\ collars} - CO_{2\ flux\ from\ 35\ \mu m\ collars}$$

$$CO_{2\ mycorrhizae} = CO_{2\ from\ 35\ \mu m} - CO_{2\ microbes}$$

Results for this partitioning are expressed as a percentage of total soil respiration. For example:

$$\%CO_{2roots} = \frac{P_{roots}}{P_{roots} + P_{microbes}} \times 100$$

Due to difficulties with these data (see Results), we focused our analysis on the $CO_{2\ soil}$ flux, which is referred to as 'intact', due to these measurements coming from unmanipulated columns of soil. At the end of this portion of the experiment (May 2018) soil inside the mesh cylinders was sampled using a 5.5 cm diameter corer to 30 cm depth. Analysis of these cores is pending, but will include total organic C, N, and root content at depth (0-5, 5-10, 10-20, & 20-30 cm) to determine the effectiveness of the mesh at excluding roots as well as the effect the mesh had on soil C and N pools and how these contributed to measured fluxes.

Automated Soil Respiration Measurements

In order to obtain fine temporal scale data on soil respiration, in May of 2015 we established automated soil respiration chambers (Figure 1) beneath tree canopies and in open grassland areas of the N only and control plots (n = 4 per unique habitat and treatment combination). These chambers measure soil respiration every half-hour using a Li-820 gas analyzer (Li-Cor Biosciences, Lincoln, NE, USA). Fluxes were processed for periods corresponding to the SP campaigns using a fixed 40 s lag-time via the same R package, *RespChamberProc*. Due to equipment failures and the remoteness of the site leading to difficulty in maintaining the chambers, several long gaps exist in this data. Gap-filling was done by fitting a random-forest model (Breiman 2001) with time of day, air temperature, soil temperature, precipitation, vapor-pressure deficit, soil moisture, and day length (to maintain seasonal trends). The final number of records used for analyses was approximately 76 per day

Figure 1. Automated soil respiration chamber in closed position. Chambers were installed in May 2015 in open grassland and under canopy habitats of the control and +N plots (n = 4, per unique habitat & treatment combination).

Figure 2. Modified 1 L glass jars used for 24 h incubation of washed plant roots (left) and sieved soil (right) from 0-5 cm depth. CO_2 from headspace gas was collected for determination of $\Delta^{14}C$ signatures.

across treatments and campaigns with an average of 420 observations for a single campaign, ranging from 68 to 1011 observations.

^{14}C Partitioning

In addition to the physical respiration partitioning installation, samples were collected to determine the radiocarbon signature of soil respiration and its autotrophic and heterotrophic components. Provided sufficient spread in these two end-members, a simple weighted-average equation can be solved to determine their contribution to total soil CO_2 flux. Samples were collected in May 2018 on two consecutive days with similar weather conditions. Two complete sets of replicates were collected on each day. For each SP in the control and +N plot a 10x10x5 cm volume of soil was excavated. The green component of vegetation was cut away and plant roots and coarse components were removed from the mineral soil (2 mm sieve). Roots were washed with distilled water and patted dry. Then roots and soil were placed in two separate 1 L mason jars with a customized lid which allowed for gas sampling without removal (Figure 2). While this sampling was underway, a sealed chamber was placed over the intact soil respiration collar for the same station and left in place for 30 minutes. Once 30 minutes had passed, tubing on the chamber was connected to a portable gas pumping device which first directed sampled air through a water-trap (magnesium chloride), then removed CO_2 using a molecular sieve trap containing 3 g of zeolite. Chamber gas was pumped for 10 minutes. The 1 L mason jars containing roots or soil were left in a shaded place for 24 h and the following day extracted using the same set-up as above with 5 minutes pumping time. Zeolite traps were extracted at 500˚C and the CO_2 graphitized for running on a 3MV Tandetron Accelerator (HVEE, Amersfoort, Netherlands) at the ^{14}C analytical facilities in Jena, Germany (Steinhof et al. 2017).

In order to quantify the respective role of plant roots and soil microbes in total soil respiration, the flux of CO_2 from soil was modeled by the following equations:

$$CO_{2\,soil} = CO_{2\,roots} + CO_{2\,microbes}$$

$$\Delta^{14}CO_{2\,soil} = (P_{roots} * \Delta^{14}CO_{2\,roots}) + ((1 - P_{roots}) * \Delta^{14}CO_{2\,microbes})$$

where P_{roots} is the portion of total soil respiration that comes from plant roots. The $\Delta^{14}CO_2$ of soil, roots, and microbes were measured and the equation solved for P. Results for this partitioning are expressed as a percentage of total soil respiration. Ex:

$$\%CO_{2roots} = \frac{P_{roots}}{P_{roots} + P_{microbes}} \times 100$$

Ancillary Data

Ancillary data on soil C, N, and P pools were collected from the immediate vicinity of SP's by pooling three 0-5 cm deep cores collected during each sampling campaign (Table 1). These three cores were placed into one sample bag per SP stored at 4˚C until processing (typically 24 h or less). Soil was sieved (2 mm) and 20 g subsamples were extracted by shaking for 1 h in 100 mL of 2 M KCl (for inorganic N) or 0.5 M $NaHCO_3$ for (phosphate-P). The supernatants were filtered using Whatman no. 1 (N) and no. 42 (P) filter papers pre-leached with 30 mL distilled water. Colorimetric analysis of extracts for ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{-3}) was conducted using standard methods on a Lachat QuickChem 8500 (Lachat Instruments, Hach Company, Loveland CO, USA). Subsets of these data are reported elsewhere (Nair et al., 2019). Additional 7 g subsamples were oven dried for 48 h (until mass loss ceased) at 45˚C to determine gravimetric water content which was later used for back-calculating extract values into a per mass dry soil form.

After final dry mass was recorded for gravimetric water sub-samples, this dry soil material was ground to a fine powder using a ball-mill (MM 400 Mixer Mill, Retsch Inc., Haan, Germany). A 250 mg

Table 1. Timeline of fertilization, installation, and sampling at the site. Soil sampling points (SP's) refer to respiration collars from the partitioning experiment in 4 plots (control, +N, N+P, & +P) and sampling here includes collecting data on soil C, N, and P pools. Chambers are the automated chambers making half-hourly measurements of soil respiration in the +N and control plots only.

When	What
December 2014	Application of P fertilizer in N+P and +P plots (50 kg P ha^{-1})
March 2015	Application of N fertilizer in N+P and +N plots (100 kg N ha^{-1})
May 2015	Installation: Chambers
December 2015	Reapplication of P fertilizer in N+P and +P plots (25 kg P ha^{-1})
March 2016	Reapplication of N fertilizer in N+P and +N plots (50 kg N ha^{-1})
December 2016	Installation: SP's
March 2017	SP Sampling, +N and Control ^{14}C
May 2017	SP Sampling
August 2017	SP Sampling
November 2017	SP Sampling
March 2018	SP Sampling
May 2018	SP Sampling, plus coring
March 2019	De-installation: SP's

subsample was weighed for total C and N analysis (Vario Max, Elementar Inc., Langenselbold, Germany). Soil pH was determined for samples collected in December 2016 (during respiration partitioning cylinder installation) using a 1:1 distilled water slurry. All samples had a pH below 6 (Table 2), so we assumed that carbonates play a negligible role in soil C stocks and that total C equals total organic C. More extensive sampling of the treatment plots occurred in May 2018, increasing the sample size to n = 8 for open grassland habitats, and n = 6 for under tree canopies, by adding additional sampling points from randomly selected locations throughout each plot.

Table 2. Surface soil (0-5 cm) pH from four nutrient addition treatments and two distinct habitats of a dehesa (n = 4-6) as determined using a 1:1 slurry with 0.01 M $CaCl_2$. Values vary significantly by habitat but not by treatment.

Plot	Habitat	mean	±	sd
Control	Open Grassland	5.86	±	0.2
	Under Canopy	5.60	±	0.3
N added	Open Grassland	5.66	±	0.3
	Under Canopy	5.24	±	0.3
N+P added	Open Grassland	5.79	±	0.2
	Under Canopy	5.45	±	0.4
P added	Open Grassland	5.56	±	0.2
	Under Canopy	5.20	±	0.6

Gap-filling of soil temperature data

For filling temperature data gaps in the manual soil respiration dataset, two additional data streams from the site were utilized; soil temperature probes associated with the eddy-covariance towers, and soil moisture data collected during SP campaigns as part of the ancillary data. Each eddy covariance tower has two soil profiles per habitat type equipped with temperature probes at various depths (12 profiles in total across all treatments). We utilized all data from the 2 and 5 cm depths, which corresponded temporally with our SP sampling campaigns and had less than 5% missing values. This temperature data, as well as laboratory determined soil moisture, previously collected soil temperature data from the campaigns, habitat, nutrient addition treatment, time of day, season, and year were used as predictors to train a Random Forest model which filled missing temperature data (Breiman 2001). The performance of this model was good, with an R^2 of 0.953 and standard deviation of the predictions of 1.07˚C, obtained via cross-validation.

Statistical analysis

Basal respiration and temperature sensitivity analysis

To determine if there are effects of habitat and nutrient addition treatment on basal respiration, Rb (soil respiration at 10˚C), and temperature sensitivity (Ea), we fitted the soil respiration and temperature data to the Arrhenius model (Davidson and Janssens 2006). We then analyzed the variance

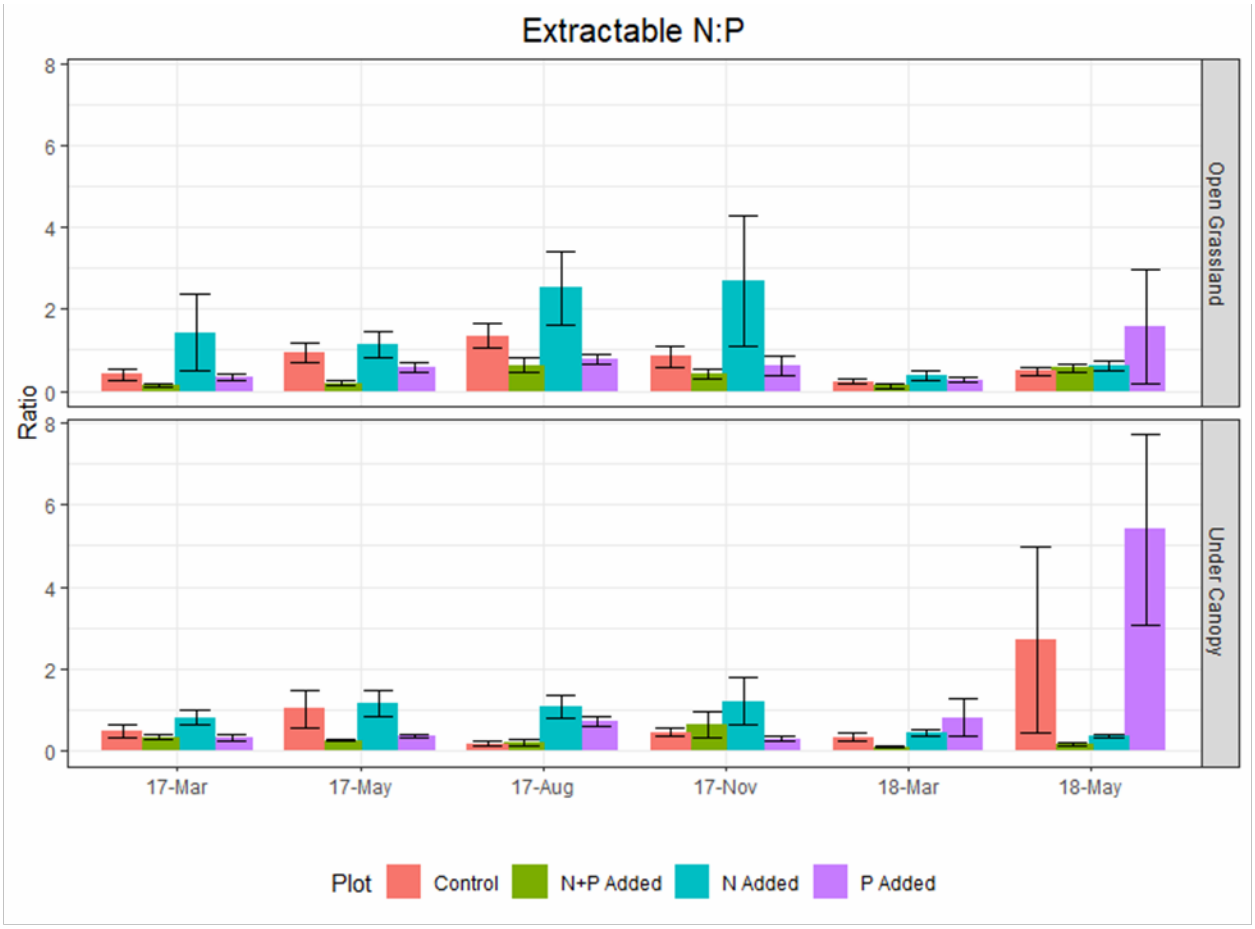

Figure 3. Extractable N:P ratio from two dehesa habitats over six sampling campaigns. Values are means ± standard error, n = 4, of the ratio of extractable inorganic N (2 M KCl) to extractable phosphate-P (0.5 M $NaHCO_3$). There is a significant effect of campaign and of nutrient addition treatment ($p < 0.001$).

in calculated Rb and Ea by campaign, nutrient addition treatment, and habitat using both a fixed-effects model (manual measurements) and a mixed-effects model (automated chamber-based data). The contrast between these two data collection methods was necessary due to the differing amounts of data available for the periods of interest. This also provided a unique opportunity for insight into methodological trade-offs because manual measurements are stronger in capturing spatial variability and automated measurements have greater temporal resolution. For manual measurements, to increase comparability to the analysis of automated chamber data, separate models were run using only the control and +N data, as well as all four nutrient addition treatments. Fluxes from intact soil and root in-growth mesh (400 µm) were used. For the manual measurements a random effect for only two measurements per season and treatment/habitat class was estimated not to be significantly different from zero, hence we report the results of the fixed-effects model. In this model, the fixed effects of campaign, as well as nutrient addition treatment, habitat, and their interaction were additive. For

automated data, we allowed a random effect of individual respiration manual measurements on Rb, but not Ea, because although spatial heterogeneity may affect the starting value of soil respiration, it should not affect its sensitivity to temperature changes. This analysis was conducted in R using R studio version 3.6.0 and the non-linear fixed effects function *gnls* and the mixed effects function *nlme*, both from the *nlme* package (Pinheiro et al. 2017, R Core Team 2019).

Mixed-effects model

Differences amongst habitat, nutrient addition treatments, and campaign in soil respiration values from manual and ancillary data were analyzed in R. We used a mixed effects model with nutrient addition treatment (control, +N, +P, and +N+P), habitat, and campaign as fixed effects, and as above the interaction between habitat and treatment was included. SP was included as a random effect. Because we sampled a relatively short time series (6 campaigns), we treated campaign as a fixed factor without a time series autocorrelation-term in the models. Data were tested for normality using visual inspection of histograms and q-q plots. If a transformation was necessary, we applied Tukey's Ladder of Powers to maximize the Shapiro-Wilk's W statistic of the distribution using the function transformTukey() from the package *rcompanion* (Mangiafico 2019). Using the function lmer() from the package *lmerTest* (Kuznetsova et al. 2017), analysis of variance (ANOVA) was computed. Using the function emmeans() from a package by the same name (Lenth 2019), Tukey's Honest Significant Difference post-hoc comparisons on estimated marginal means were carried out. Degrees of freedom were calculated using the Satterthwaite's method for ANOVAs and the Kenward-Roger method for Tukey tests. Effects and comparisons were considered statistically significant at $p \leq 0.05$.

Results

Treatment effects on soil N:P stoichiometry

Extractable N:P varied by campaign and was statistically lower in the +N+P treatment compared to the others when comparing across campaigns and habitats (Figure 3). This was driven by greater extractable P in the +N+P treatment, rather than lower extractable N. When looking at extractable P on its own, the +N treatment had the lowest values, followed by the control and the +P, with +N+P being the highest. There was comparatively little variation in extractable N. There were generally larger extractable pools under tree canopies, but lower N:P ratios (Figure 3). Data from the more extensive sampling campaign in May 2018 showed that even with higher sample sizes, extractable N:P was highly variable,

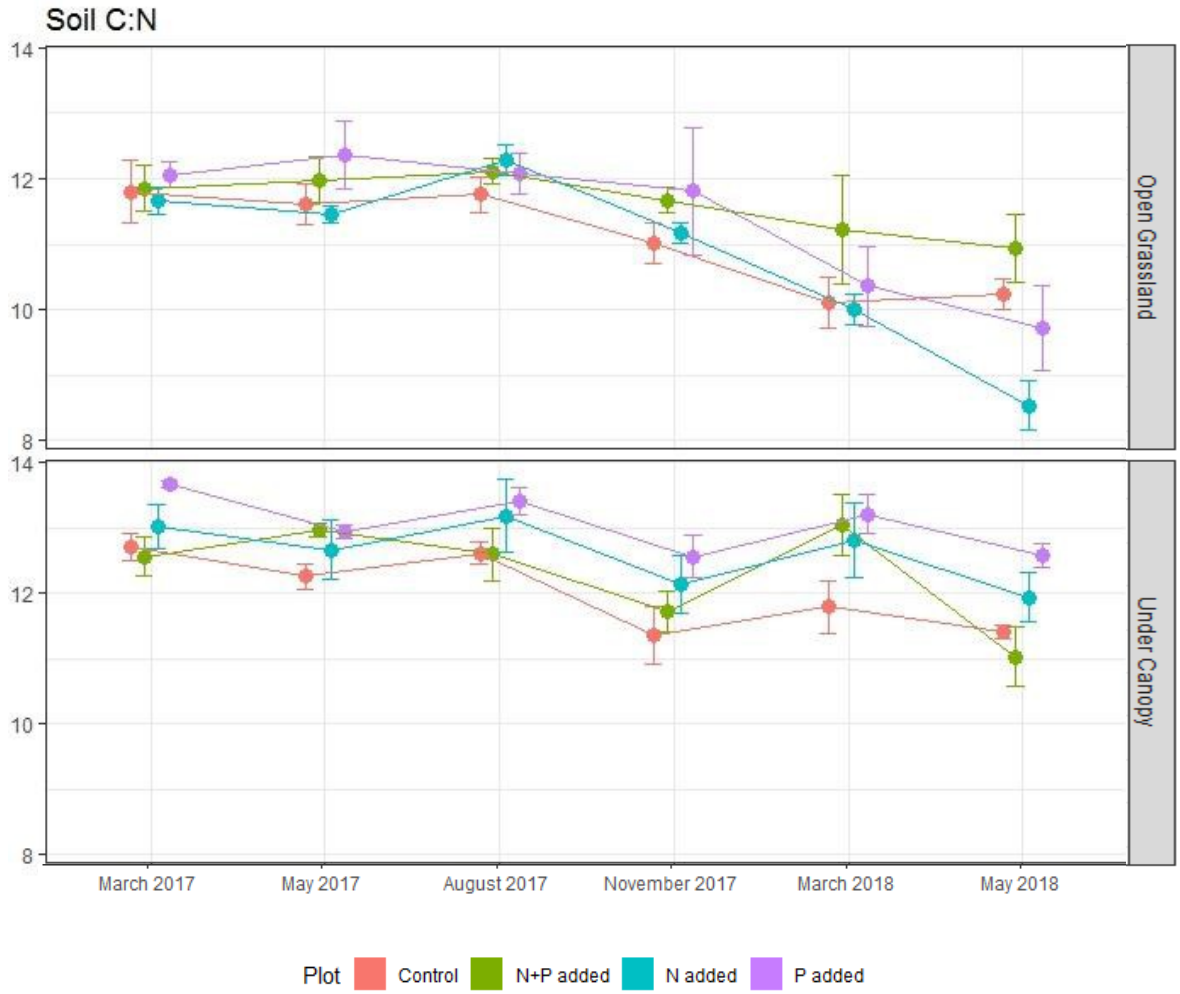

Figure 4. Soil C:N ratio from two dehesa habitats over six sampling campaigns. Values are means ± standard error, n = at least 4. There is a significant effect of campaign ($p < 0.001$) and habitat ($p < 0.05$).

and while there was no significant difference between habitats, the +P plot had significantly higher N:P than other nutrient treatments and this was especially pronounced in the under canopy samples ($p < 0.05$ for habitat*treatment interaction).

Soil bulk C:N tended to be lower in the open grassland than under canopies, averaging 11:1 vs 13:1 for the duration of the experiment across all treatments. Values fell noticeably throughout the period of data collection, dropping on average 1 point for both habitats (Figure 4). This drop was mostly due to decreasing C pools.

Treatment effects on soil respiration

Soil respiration from intact-soil manual measurements was consistently greater under tree canopies in the control treatment (Figure 5), but the nutrient additions significantly altered this

relationship (habitat*treatment, $p < 0.05$). Addition of N or P alone increased soil respiration in the open grassland habitat to be statistically similar to that under canopies. Within habitat, the +N plot had significantly greater respiration rates than the control in the open grassland, but neither differed from the +N+P or +P (this differs if all in-growth meshes are considered, both +P and +N diverge from +N+P and control). There were no consistent or significant differences amongst nutrient addition treatments in the under canopy habitat.

Temperature sensitivity (Ea) and basal respiration (Rb)

When comparing campaign data from manual measurements to data collected by the automated soil respiration chambers in the same periods, the fluxes were of similar magnitudes, generally between

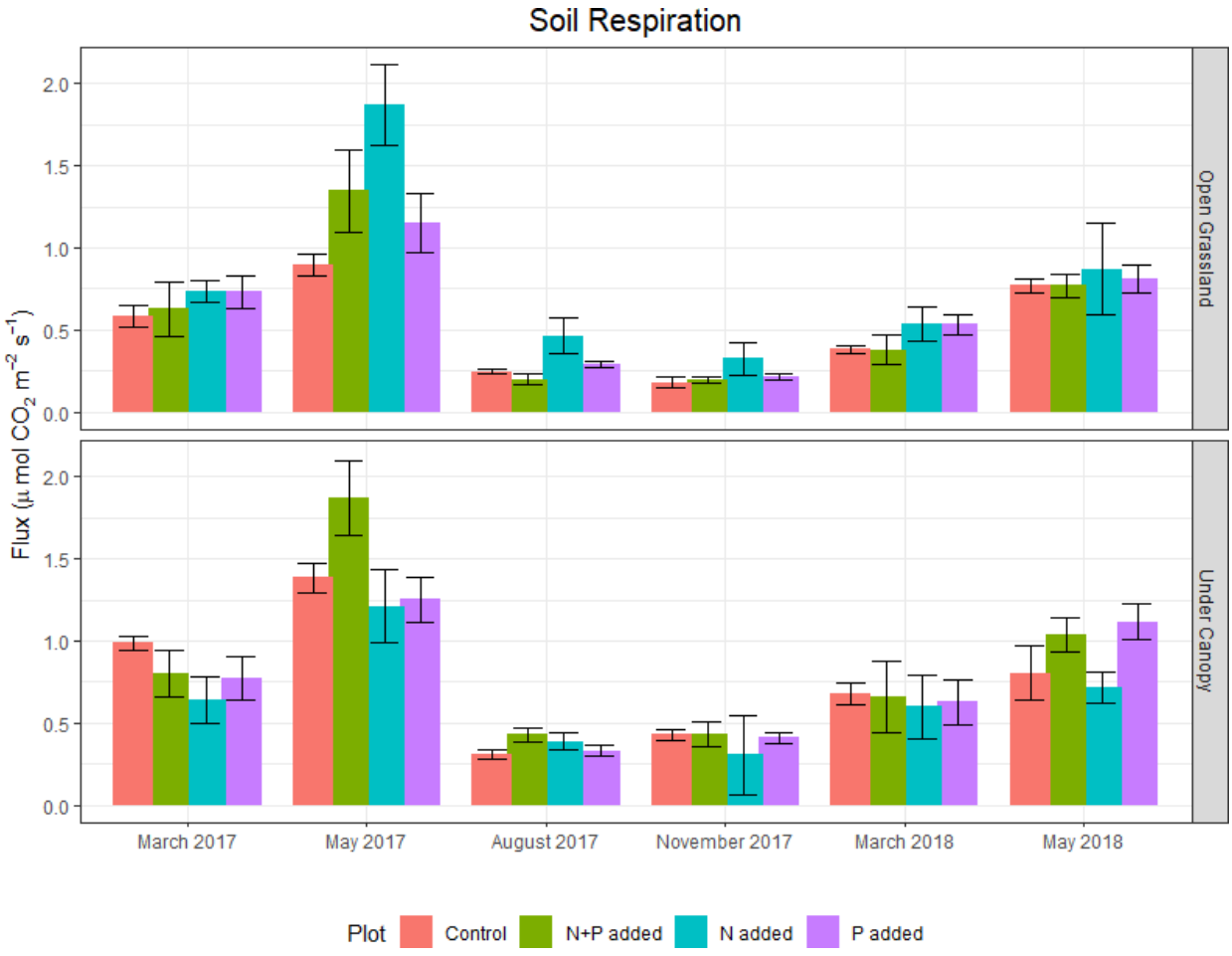

Figure 5. Soil CO_2 efflux (respiration) from intact soil collars from two dehesa habitats over six sampling campaigns. Values are means ± standard error, n = at least 4. There is a significant effect of campaign ($p < 0.001$) and a significant interaction between nutrient addition treatment and habitat ($p < 0.05$) with N addition significantly increasing respiration relative to the control in the open grassland, but not under canopies.

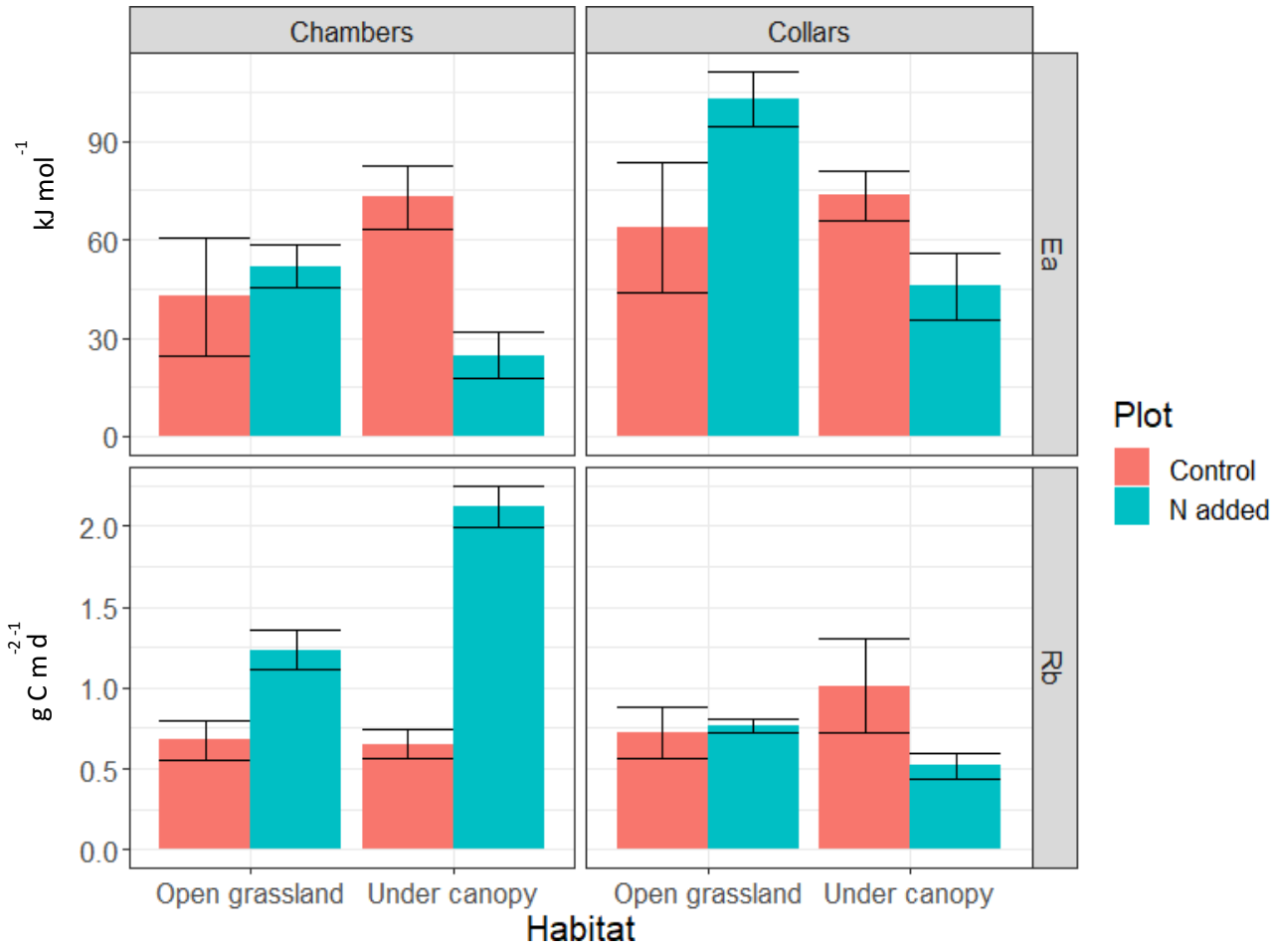

Figure 6. Comparison of two data collection methods for assessing the response of temperature sensitivity (Ea, kJ mol^{-1}) of respiration and basal respiration (Rb, soil respiration at 10˚C, g C m^{-2} d^{-1}) between habitats and with N addition. Values are over-all averages of analyses from soil respiration collars ('Collars') vs automated chambers ('Chambers').

0.5 and 3 g C m^{-2} d^{-1}. The largest fluxes were measured on May 2017 for both methods, with max values slightly above 4 g C m^{-2} d^{-1} for automated and slightly below 4 g C m^{-2} d^{-1} for manual measurements. However, the results of our Arrhenius models looking at basal respiration and temperature sensitivity of soil respiration in response to habitat and N addition differed in their outcomes (Figure 6), very likely due to their differing abundances of data and methodological trade-offs. Rb and Ea values were similar between automated and manual measurements in the control plot for both habitats. However, the effect of N addition differed for the two measurements, especially with regard to Rb (Figure 6). Rb significantly increased with N addition in both habitats when analyzed using the automated data. With the manual measurements, there was no change in basal respiration in open grassland and a modest, but statistically significant, decrease under canopies. Model output from the two methods had better agreement when

looking at N addition effects of Ea. Both methods showed modest increases in sensitivity with N addition in open grassland, and decreases under canopies.

Table 3. Coefficients from Arrhenius model fitting basal respiration (Rb., g C $m^{-2}d^{-1}$) and temperature sensitivity of respiration (Ea., kJ mol^{-1}) to fluxes measured from intact and root-growth respiration collars in control and 3 different nutrient addition treatments in two habitats (n = 4-8). The intercept represents fluxes from the March 2017 campaign in open grassland of the control treatment and subsequent values are additive. UC stands for under canopy.

Coefficient	Value	Std. Error	p-value
Rb. (Intercept)	0.78	0.08	< 0.001
Rb. N added	0.07	0.07	N.S.
Rb. NP added	0.05	0.08	N.S.
Rb. P added	0.09	0.07	N.S.
Rb. Habitat: UC	0.33	0.06	< 0.001
Rb. N added + UC	-0.24	0.09	< 0.01
Rb. NP added + UC	0.10	0.11	N.S.
Rb. P added + UC	-0.05	0.11	N.S.
Ea. (Intercept)	-150.07	90.65	N.S.
Ea. N added	28.06	8.41	< 0.001
Ea. NP added	17.63	9.22	0.06
Ea. P added	5.11	9.54	N.S.
Ea. Habitat: UC	3.03	8.48	N.S.
Ea. N added + UC	-10.53	11.37	N.S.
Ea. NP added + UC	-8.51	11.87	N.S.
Ea. P added + UC	-5.95	12.47	N.S.

Models were robust within their limitations, meaning that although inclusion of random effects would be over-fitting of the manual-based date, trial runs that included such terms yielded similar statistical results. Due to the greater amount of data available from automated measurements, we were able to bootstrap the data used in the Arrhenius model, giving a very high degree of confidence in the data presented. We refitted the mixed model to bootstrapped samples (draw random observations from the original automated data with resampling) 60 times. From the resulting bootstrap-sample of habitat and treatment effects on Rb and Ea, we could estimate statistics such as the standard deviation and 95% confidence bands.

When looking at all nutrient addition treatments (manual data only, Table 3), we see that Rb and Ea were not strongly effected by P addition, whether or not that P was accompanied by N addition. The only nutrient addition treatment to significantly change Rb was N addition under tree canopies (Table 3, see also Figure 6). Ea of soil respiration was increased with N addition in open grassland (as above), but no nutrient addition treatments effected this parameter under canopies (Table 3). There was a near-significant increase of the Ea in open grassland with +N+P addition ($p = 0.06$, Table 3).

Respiration Partitioning

Results from in-growth cores

Our physical partitioning experiment was unable to consistently distinguish between autotrophic and heterotrophic respiration (Figure 7), this is manifest in the tendency for fluxes from root in-growth cores (400 µm mesh) to be lower in magnitude than those from the root and hyphal exclusion cores (4 µm mesh). However, it is worth noting that our underlying assumptions do hold true in some cases and that these vary by habitat. In the open grassland, our calculated root respiration was measurable in both

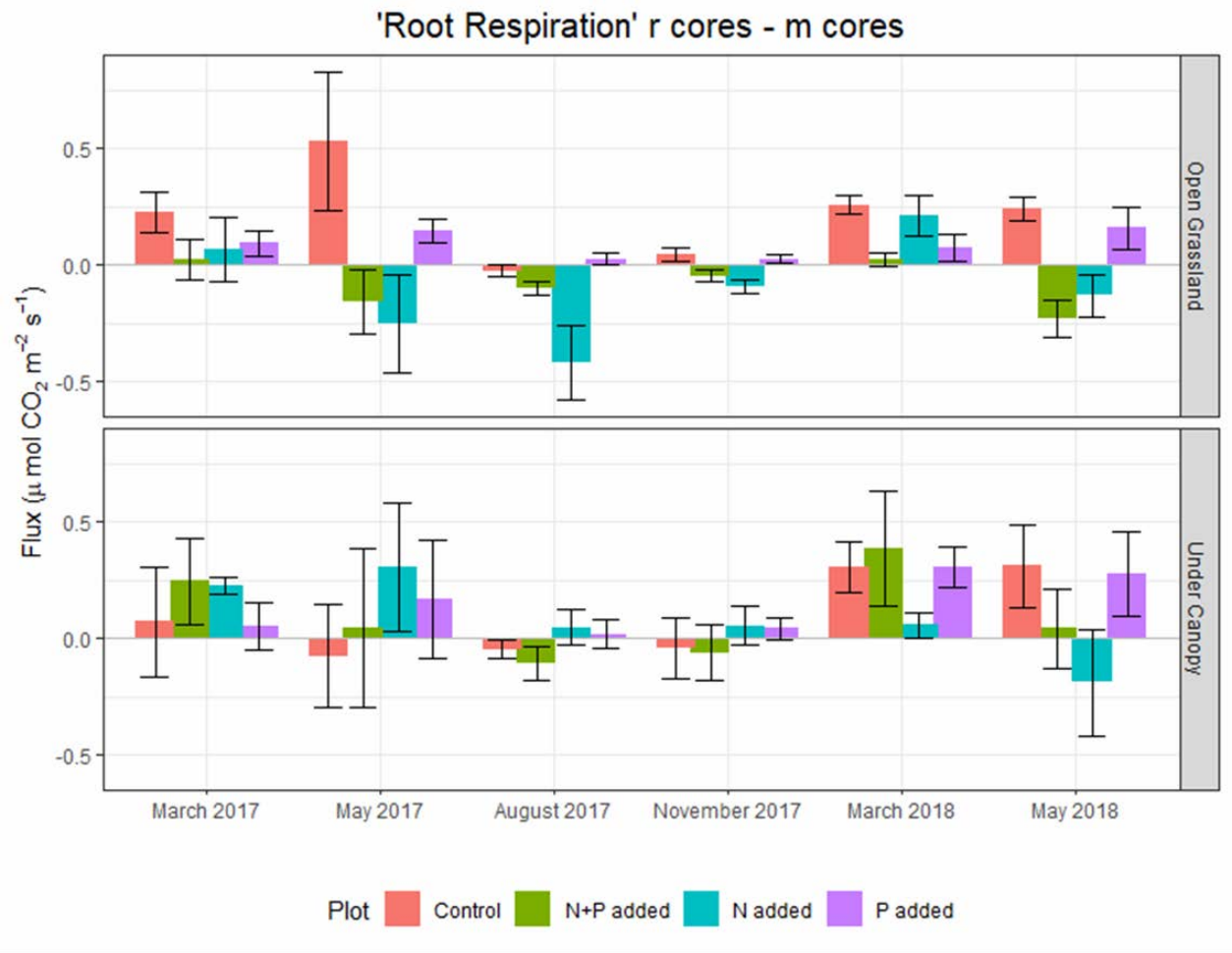

Figure 7. Absolute difference in soil CO_2 efflux from root in-growth (400 µm) and no in-growth (4 µm) mesh cylinders in two dehesa habitats over six sampling campaigns, n = at least 4. These data were originally meant to calculate the flux attributable to plant roots, but the frequency of negative values indicates that the physical partitioning did not succeed.

spring growing seasons for both the control and +P treatments, while under canopies it was highly variable between the two springs. Additionally, values under canopies tended to be closer to zero in the dry and dormant (see November '17, Figure 7) seasons in all treatments, but in the open grassland these values went strongly negative in the dry season with either N addition type (most obvious in +N) and stayed negative into the dormant season.

Results from radiocarbon signatures

Our radiocarbon data were also unable to consistently distinguish between autotrophic and heterotrophic respiration. We were, however, able to determine the $\Delta^{14}C$ signatures of individual end members, which varied significantly by an interaction between habitat and treatment ($p < 0.05$). Values of soil, roots, and respired C tended to be lower under canopies in the N added plot, but did not differ by treatment in the open grassland habitat (Table 4). Combining habitats and nutrient addition treatments,

Table 4. $\Delta^{14}C$ of respired CO_2 from the intact soil system, plant roots, or sieved mineral soil from the control and N addition plots and two distinct habitats of a dehesa (n = 4). Values vary significantly by an interaction between habitat and treatment ($p < 0.05$).

Type	Plot	Habitat	mean	±	se
Intact Soil	Control	Open Grassland	6.22	±	5.9
		Under Canopy	9.60	±	11.7
	N added	Open Grassland	9.10	±	3.9
		Under Canopy	8.90	±	5.4
Root	Control	Open Grassland	-14.00	±	13.2
		Under Canopy	11.05	±	5.6
	N added	Open Grassland	-2.75	±	3.4
		Under Canopy	-12.45	±	3.1
Mineral Soil	Control	Open Grassland	4.20	±	4.7
		Under Canopy	10.03	±	9.5
	N added	Open Grassland	6.78	±	2.9
		Under Canopy	2.97	±	4.2

respired CO_2 had the highest delta value (8.24 ± 2.9, mean ± standard error), followed by mineral soil (6.19 ± 2.9), then plant roots (-3.90 ± 3.9). The first and last ecosystem components differed significantly from each other ($p < 0.05$), indicating a significant contribution of heterotrophic respiration to soil CO_2 flux.

Discussion

We set-out to determine the effect of habitat and nutrient addition on soil respiration, Rb, and Ea. Our experimental design utilized a previously established fertilization experiment focused on shifts in ecosystem processes with N and P availability. Below we report on soil respiration and related measures with an emphasis on the effect of habitat (under canopy vs open grassland), and N:P ratio of the nutrient addition treatment. We expected that soil under canopies would have reduced respiration in response to nutrient addition (Janssens et al. 2010), that open grasslands would have the opposite (Peng et al. 2011), that Ea of respiration would be greater under canopies (Davidson and Janssens 2006), but this habitat effect would reverse with nutrient addition (Conant et al. 2008).

Response of soil respiration to nutrient addition

Under tree canopy

We hypothesized (H1) that under canopy soil would have decreased soil respiration with all nutrient additions, however this effect was variable over time. The +N treatment flux tended towards lower values than the control (Figure 5). Our Arrhenius model output was also variable, with increased Rb

under canopies with N addition based on automated-data, and the opposite based on manual measurement data (Figure 6). While May manual measurements of both years show increased respiration in the +N+P plot under canopies (Figure 5), this effect was not mirrored in our Rb analysis. It is also worth noting that soil under tree canopies had consistently higher respiration rates than soil in open grassland across all of our metrics, which is expected given that the under canopy habitat should contain the vast majority of tree roots which additionally contribute to soil respiration. Furthermore, this habitat has greater soil C content, which should support greater rates of microbial respiration as well.

The review of Janssens *et al* (2010) indicates that in 75% of the studies they surveyed, N addition had a negative effect on forest soil CO_2 flux, averaging -17%. In our study we found roughly this reduction in the growing season, with the flux being reduced by 24%. Various reasons are given for decreases; shifts in pH, decrease in belowground C flux from plants, and shifts in microbial community/activity (Janssens et al. 2010, Ramirez et al. 2012, Zhang et al. 2014, Guo et al. 2017). Changes in pH are universally known to effect soil microbes, but are unlikely here given that our soil pH data shows minimal changes due to nutrient additions (Table 2). The final hypothesis, invoking changes in microbial communities and/or activity, has peripheral support from numerous studies in other ecosystems (*e.g.*, Ramirez et al. 2012), and some data collected at the site (see Gogesch 2019 and chapter 4 of this thesis). However, the most relevant mechanism for consideration in global C cycling is decreased belowground allocation. We know from data collected by minirhizotrons and root in-growth cores at the site that root biomass has in fact increased with N addition (Nair et al. 2019). But we also know that arbuscular mycorrhizal fungi (AMF) at the site have lower concentrations of their preferred storage lipid (Gogesch 2019), indicating reduced plant investment of C in their fungal symbionts. This presents a classic case in ecology, wherein multiple mechanisms push the response of the system in different directions. It seems likely that even though root biomass increased, microbial activity (free-living and AMF) decreased sufficiently under tree canopies to result in a tendency of reduced respiration.

Open grassland

The response of soil respiration under canopies sets a sharp contrast with the second habitat type we investigated in the oak-savanna, the open grasslands. We hypothesized (H2) that open grassland would show increased respiration in response to nutrient addition. This was strongly supported for N addition, but not any other nutrient addition treatment (Figure 5) and further supported by increased Rb rates in our analysis using automated measurements (Figure 6).

We predicted this contrast between habitats because of the lower quality of the soil in the open grassland compared to under canopy. Other habitats that show increased soil respiration with N addition (the 25% from Janssens *et al.* 2010) include sites early in development or where N addition significantly increases photosynthetic rate. While +N+P and +P also tended to increase respiration in this habitat, the N addition treatment was the only statistically significantly one. We know from other work at the site that +N and +N+P increased photosynthesis and ecosystem respiration rates, specifically for the open grassland (Migliavacca et al. 2017). This supports our hypothesis that the open grassland soil respiration would be driven by more complete annual turnover of plant biomass in this habitat, with greater productivity in the fertilized areas leading to greater soil respiration (Janssens et al. 2001, Davidson and Janssens 2006). The increase in productivity is likely happening in the herbaceous layer under tree canopies as well, but the response of soil respiration is confounded by other components of the habitat there. Therefore, we believe that decreased respiration under canopy is reflective of the signature from increased root biomass respiration masked by decreased heterotrophic (and mycorrhizal) respiration. This is supported by previous work showing that the total microbial biomass under tree canopies is much higher (2-3x greater) than open grassland, meaning that their ability to influence the total soil respiration budget is greater (Gallardo 2003, Morris et al. 2019).

Ea of soil respiration and Rb rates

We hypothesized that under canopy soil would show greater Ea, due to larger SOM pools (Gallardo 2003), indicating that a wider variety of chemical species are present (Davidson and Janssens 2006). We measured higher values of Ea for under canopy soil in both manual and automated-chamber measurements, but the effect was relatively small and not statistically significant. Instead we found that Ea was affected by the +N treatment in both habitats, and not by any other nutrient addition treatments. Arrhenius models using either manual or automated measurements agree about the direction of change of Ea with N addition, although the magnitudes for the two different habitats differ (Figure 6). Ea increases in open grassland with N addition, supporting our final hypothesis, and decreases under tree canopies under the same conditions. It is believed that increased Ea reflects the decomposition of more recalcitrant (more chemically complex) SOM (Davidson et al. 2012). In general, Ea should increase with increased ecosystem productivity (something known to have occurred in the +N treatment; El-Madany et al. 2018, Luo et al. 2018) due to more plant C becoming available to soil microbes, facilitating the decomposition of more chemically complex SOM (Conant et al. 2008). There is some evidence that this is similar to the rhizosphere priming effect, which also has a positive influence on Ea (Zhu and Cheng 2011). However, we

see the opposite effect on under canopy Ea, meaning that sensitivity values there may be more related to the response of tree root respiration. which is expected to decrease with N addition (Janssens et al. 2010). Alternatively, increased plant productivity under canopies might not have led to increased decomposition of chemically complex SOM due to differing microbial communities between the two habitats (Lai et al. 2014, Gogesch 2019), meaning that decreased Ea in the +N treatment compared to the control is simply due to soil microbes using more readily decomposed substrates.

Respiration partitioning

Because soil respiration is comprised of autotrophic and heterotrophic components, which can respond differentially to nutrient addition, we had hoped to partition soil respiration fluxes between plant roots and free-living soil microbes. However, our attempts at partitioning were ineffective (Figure 7, Table 4), likely due to high spatial heterogeneity. Future analysis of soil cores may give additional information as to why the manual measurements partitioning did not work. Comparison of root in-growth respiration to microbial-only respiration indicates that partitioning most closely resembled expected patterns in low N:P (+P plot) or unaltered N:P (control, Figure 7).

Consideration of automated vs manual measurements

We were surprised by the different results of the two datasets (manual vs automated) for the Arrhenius model (Figure 6). While there is good agreement in terms of the effect of habitat and N addition on Ea (Figure 6, upper panel), a divergence occurs in terms of the effect of N addition on Rb (Figure 6, lower panel). For automated measurements Rb increases with N addition (more so under canopy than in open areas), while for manual measurements there is a modest (but statistically significant) decrease under canopies and no change in the open grassland. Fewer observations are included in the analysis using manual measurements, which would make this analysis more sensitive to the range in temperature encountered at each sampling date and possibly contributed to some of the observed discrepancies for Rb and Ea. However, if we consider both models to be robust, then spatial heterogeneity and sampling error are possible reasons for the differences between the analyses.

Spatial heterogeneity is the rule, rather than the exception, for many soil properties. As more data are collected and analyzed within the context of this large-scale field experiment, we are realizing that heterogeneity is exceptionally high at our site (Nair et al. 2019). Knowing this, it is possible that our spatial replicate number of 4 for the automated chambers, and 8 for soil respiration manual measurements (when both intact and root-ingrowth measures are included), is likely insufficient to capture the full spread

and population mean of soil respiration measures. Furthermore, in addition to low spatial replication, the automated chambers in the under canopy habitat of the N addition plot are all located under the same oak tree. Therefore, data from this data set are potentially specific to the response of that individual tree (assuming the data are dominated by root-respiration). The collars for measuring under canopy soil respiration in the N addition plot manually were split between two different trees, which is admittedly only a modest improvement. The final contributing factor for accurately capturing the effect of N addition on soil respiration has to do with how the fertilizer was applied. A GPS equipped tracker distributed fertilizer pellets throughout the target area, and an image showing this GPS track was used to inform where the soil respiration collar for manual measurements would be installed. In contrast, the automated chambers require a constant power source and therefore had to be installed under the one tree in the plot sufficiently close to a power source to allow for that. Despite these limitations, the higher resolution of the data from automated chambers in time (also capturing a greater range of temperatures) means that our model results from automated data are likely more robust than those from manual measurements in terms of correctly capturing Ea and Rb for their specific spatial locations. Additional data would need to be collected at both high spatial and temporal resolution in order to fully understand the differences between the two analyses.

Conclusion

Collectively our results indicate that soil respiration in this oak-savanna is more sensitive to increases in the N:P ratio of soil nutrients than to increased availability of both N and P (+N+P treatment) or decreases in the N:P ratio of soil nutrients (+P). This implies that the ecosystem is more limited by N than by P, and will be sensitive to N deposition due to anthropogenic inputs. Our results also indicate that habitat within the oak-savanna is a critical modifier of how soil respiration as well as Rb and Ea are affected by nutrient addition. Open grassland areas represent 80% of the surface cover in this oak-savanna, and therefore the response of this habitat is more representative of the ecosystem as a whole. The lower-nutrient, more annual-grassland like open areas were consistently more strongly effected by increases in soil N:P availability than the higher-nutrient under canopy habitat. These results indicate that SOM content is an important predictor of how nutrient addition will affect soil respiration.

Acknowledgements

This work is the result of an intensive collaborative effort with special thanks for tireless help in the field going to Mónica Lorente, Adrian Mateos, Enrique Juárez, and Yonathan Cáceres Escudero.

Richard K. F. Nair and Andrew M. Durso offered support and advice at many critical moments. The team from Freiland; Martin Strube, Martin Hertel, and Olaf Kolle, made this work possible and infinitely more enjoyable. Martin Göbel's assistance was instrumental for extracting gas samples for radio carbon analysis. The authors thank the Alexander von Humboldt Stiftung for financial support of the MaNiP project through the Max Planck Research Prize to Markus Reichstein. Thanks also goes to Kirsten Küsel, Markus Reichstein, and Susan Trumbore for stimulating discussion of the data. The authors declare no conflict of interest.

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Chapter 3

esa ECOSPHERE

Fate of N additions in a multiple resource-limited Mediterranean oak savanna

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Citation: Morris, K. A., R. K. F. Nair, G. Moreno, M. Schrumpf, and M. Migliavacca. 2019. Fate of N additions in a multiple resource-limited Mediterranean oak savanna. Ecosphere 10(11):e02921. 10.1002/ecs2.2921

Abstract. Mediterranean oak savannas, such as Spanish *dehesas*, are multiple resource-limited ecosystems found in semiarid regions which are key contributors to interannual variability of the global carbon (C) budget. Interactions between nitrogen (N) and phosphorus (P) cycles are expected to play a major role in overall ecosystem function as anthropogenic N deposition shifts ecosystems from N to P limitation, leaving unknown how increased N availability might influence C uptake. Therefore, the fate of N additions in dehesas is important for understanding global C cycling. Using a ^{15}N tracer experiment within fertilized (N or N + P) plots of a Holm oak dehesa, we tested the effects of ecosystem spatial heterogeneity (habitat), P addition, and time on the fate of added N. We expected that open pasture areas would retain more of the added N in biological components due to greater N limitation, that the addition of P would enhance N retention in biological components relative to N alone, and that added N would shift from being within the microbial biomass immediately after addition to being predominantly within plants at the beginning of the following growing season. We found that open pasture plots with N only had the greatest label recovery seven months after the start of the experiment, supporting the idea that open pasture was more N-limited than under-canopy areas. However, soil was the largest sink for added N, regardless of habitat, treatment, or time. Our results suggest that abiotic fixation of N may play an important role in modifying the effects of N deposition in dehesas.

Key words: ^{15}N; dehesa; microbial N; phosphorous; stoichiometry; tree–grass.

Received 16 August 2019; accepted 29 August 2019; final version received 1 October 2019. Corresponding Editor: Jason B. West.

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INTRODUCTION

Atmospheric nitrogen (N) deposition causes a variety of downstream effects on ecosystems, ranging from increased productivity to groundwater contamination (LeBauer and Treseder 2008, Schlesinger 2009, Schulte-Uebbing and de Vries 2018). One major consequence of N deposition is a potential shift in the biological availability of N relative to phosphorus (P) because of differential deposition rates from anthropogenic activity, driving ecosystems from N into P limitation (Peñuelas et al. 2012). The consequences of N:P imbalances are largely unknown (Sardans et al. 2012), especially in the context of how such imbalances might affect ecosystem N allocation.

In their 2011 review, Pardo et al. found that ecosystems dominated by low-biomass components (e.g., grasslands, desserts) are more sensitive to N deposition than ecosystems dominated by high-biomass components (e.g., forests) due to differing generation times and buffering abilities. Shorter generation times lead to faster responses at the community level, which, along with lower biomass, means small changes in ecosystem N availability can result in large

changes in individual biomass components (Pardo et al. 2011). This contrast in sensitivity makes predicting the response of mixed tree–grass ecosystems to shifts in N and P availability difficult (Sardans et al. 2012). Tree–grass ecosystems are distributed globally, making up roughly one-third of the terrestrial land cover (Di Castri 1991, Mistry and Beradi 2000, Hanan and Lehmann 2010). Many of these mixed cover ecosystems are located in semiarid regions which have recently received heightened attention as key players in the interannual variability of the global C budget (Ahlström et al. 2015). Increased N availability has the potential to increase C uptake of such systems, but it is not yet clear if P availability might limit this potential. Therefore, shedding light on the linkages between C, N, and P cycles is of growing importance.

Dehesas are a type of tree–grass ecosystem comprised of open oak woodland with an herbaceous layer consisting mostly of annuals (typically) or in some cases crops. They are analogous to the Portuguese *montado*, other Mediterranean wood pastures (den Herder et al. 2017), and the Californian oak woodlands of the United States (Mistry and Beradi 2000). Dehesas are multiple resource-limited ecosystems, being limited by nutrient availability early in the growing season and water later in the growing season (García and Mata 2000, Moreno 2008). This is due to the relatively nutrient-poor parent material of the soil and the extremely dry summer conditions typical of the Mediterranean climate (Olea and San Miguel-Ayanz 2006, Moreno 2008, Vitousek et al. 2010). Multiple resource limitation is characterized by sensitivity of the ecosystem to addition of any limiting resource (Wang et al. 2014, He and Dijkstra 2015, Wurzburger and Wright 2015). Previous work has highlighted the vulnerability of dehesas to N deposition due to differing responses of individual vegetative components (Ochoa-Hueso et al. 2013, Perez-Priego et al. 2015) and the possibility of exacerbated fire-cycles due to increased biomass, such as that seen in the equivalent Californian ecosystem (Ochoa-Hueso et al. 2011, Rivest et al. 2011). However, effects of N and P additions on N allocation and retention have not yet been studied in dehesas.

The response of dehesa systems to N deposition is further complicated by the extreme heterogeneity of the herbaceous layer and uneven distribution of soil properties (Delgado-Baquerizo et al. 2013, Moreno et al. 2013, El-Madany et al. 2018). Soils developing underneath oak canopies are noticeably enriched in nutrients and soil organic matter (SOM) compared to soils in the open pasture areas (Gallardo 2003, Howlett et al. 2011, Moreno et al. 2013), often with as much as three times greater soil organic carbon concentrations. Under tree canopies, soils tend to stay moist longer after rainfall, due to protection from solar radiation by the tree. However, when dry conditions persist, soil under the canopy is often drier than that in the open areas because of the additional water demands of the perennial oaks (Cubera and Moreno 2007, Breman and Kessler 2012, Dubbert et al. 2014). Therefore, these two spatial locations represent two distinct habitats, and the fate of N is expected to differ between them.

Much work has been done to determine how ecosystems allocate N, especially within the context of N deposition (Hart et al. 1993, Fenn et al. 2003, Templer et al. 2012). One of the most common methods is the use of a stable isotope tracer (^{15}N). But in the most recent review, ^{15}N tracer studies in mixed tree–grass ecosystems, such as dehesas, were still lacking (Templer et al. 2012). Additionally, many studies only look for short-term fates of tracers (days to weeks), which can differ from long-term N sinks (months–years). These longer timescales are especially important for tree–grass systems where the herbaceous layer is able to respond to nutrition additions much more quickly than the trees (Rivest et al. 2011). Therefore, we set out to explicitly test the effects of ecosystem (1) spatial heterogeneity (hereafter referred to as "habitat"), (2) P addition, and (3) time on the fate of added N in a dehesa.

1. Because nutrients are not evenly distributed throughout dehesa soil, biotic uptake may be faster or of greater magnitude outside of the tree canopy where the soil is of poorer quality. We expect that pasture areas will retain more of the added N in both plant tissues and microbial biomass due to their presumed greater N limitation and a more closed N cycle than underneath tree canopies.
2. Given the general trend observed of greater biomass production when both N and P are

added (Vitousek et al. 2010), we expect greater N uptake and retention in biotic components of the system (both plants and soil microbes) when P is added.

3. Immediately after application, we expect added N would be taken up mostly by microbes, because microbes are thought to be better competitors for mineral nutrients than plant roots in the short term (Kuzyakov and Xu 2013). In the dry season, we expected most of the label to accumulate in the soil, as inorganic N accumulation is often seen in arid soils during the dry period (Austin et al. 2004). In the following spring growing season, we expected that plants would obtain the majority of the remaining label, because it is the peak of plant N demand, as seedlings are actively investing in nutrient uptake (Otieno et al. 2011, Jongen et al. 2013). Overall, label recovery is expected to decrease with time due to gaseous losses and label transfer below the measured soil zone.

To test these hypotheses, we fertilized plots within a Holm oak (*Quercus ilex* L.) dehesa with either N or N + P followed by a ^{15}N label to trace the recovery of ^{15}N within the herbaceous layer vegetation and surface soil for one year. Because previous work in Mediterranean oak savannas found up to 90% of ecosystem N (not associated with tree biomass) was located in the top four cm of soil (Jackson et al. 1988), we focused on this active section of the ecosystem as we were interested in short-term competition between plants and soil microbes, rather than long-term competition strategies that might be utilized by the slow-responding oak trees (Rivest et al. 2011).

Methods

Site description

Our study site was located in a publicly accessible dehesa at Majadas de Tiétar (39°56′25″ N 5°46′29″ W) in Extremadura, Spain, 258 m above sea level. The tree density is roughly 20 trees/ha, and the site is grazed from early December to late June by cattle at an intensity of <0.3 livestock units·ha^{-1}·yr^{-1} (El-Madany et al. 2018). The herbaceous layer is a native biodiverse pasture, dominated by annual species (e.g., annual vernalgrass, *Anthoxanthum aristatum* (Boiss.) and soft brome, *Bromus hordeaceus* (L.)) and nonleguminous forbs (e.g., European umbrella milkwort, *Tolpis barbata* (L.) Gaertn.) grow in an Abruptic Luvisol and sustained by ~650 mm average annual precipitation, which falls mostly between winter and early spring. Since 2003, the site has been the location of an eddy covariance tower belonging to the FLUXNET network with the site identifier ES-LMa (Pacheco-Labrador et al. 2017, El-Madany et al. 2018, Luo et al. 2018). It is worth mentioning that while this study took place at the same site as several others, the sampling plots were well outside of the tower footprints and the experiment itself was separate from nutrient manipulation experiments reported elsewhere.

^{15}N tracer experiment

An area was selected at the site with a sufficient number of mature oak trees for our sampling scheme (about 1.3 ha) and then divided in half, one half for N addition and the other for the N + P addition treatment. Within each half, 18 sampling plots of 4 × 4 m were established (Fig. 1). On 20 March 2017, to prevent targeted grazing pressure on the sampling plots, the surrounding area (excluding the treatment plots) was fertilized using pelleted ammonium nitrate (NH_4NO_3) and monopotassium phosphate (KH_2PO_4) at a rate of 50 kg N/ha, with an additional 25 kg P/ha within the N + P treatment. Mixtures were hand-thrown, specifically avoiding the sampling plots. Sampling plots were then fertilized using pelleted fertilizer pre-weighed to create exactly the fertilization loads approximated by the hand application. N addition plots received 129 g potassium nitrate (KNO_3) and 183 g NH_4NO_3 (equivalent to 50 kg N/ha), while N + P plots received 232 g NH_4NO_3 and 176 g KH_2PO_4 (equivalent to 50 kg N/ha + 25 kg P/ha). These doses are approximately five times the current N deposition rate, with sufficient P addition in the N + P plots to maintain the ecosystem's original herbaceous layer N:P stoichiometry (Migliavacca et al. 2017). Within each treatment area, nine plots were under tree canopies and nine were in open pasture areas. For each set of nine plots, three remained unlabeled while the other six received 1.1 g of 99.9% ^{15}N ammonium nitrate (Berry & Associates, Dexter,

Michigan, USA). On 21 March, pre-weighed aliquots of labeled salt were dissolved in 2.5 L of distilled water and sprayed onto plots in 0.5-m strips using guidelines and a hand-sprayer. After the 2.5 L of labeled salt solution was distributed, the hand-sprayer reservoir was rinsed with an additional 0.5 L of water, which was also sprayed onto each plot.

While our design is technically pseudoreplicated, there are a several arguments that support its validity. First of all, spatial heterogeneity is extremely high at this site (Nair et al. 2019) and multiple assessments of ecosystem parameters (ranging from root mass, chlorophyll content of vegetation, and carbon and water fluxes) have found that variability on the scale of 1–2 m (such as our sampling plots) is equal to variability on the scale of hundreds of meters (El-Madany et al. 2018, Nair et al. 2019). Additionally, because the site is grazed during the vegetative period of the herbaceous layer, it was necessary to fertilize additional surface area to prevent preferential grazing pressure on fertilized sampling plots. However, scattering N-only plots within a N and P fertilized area runs the risk that P from the surrounding fertilization could influence perceived N-only treatment effects if added P is relocated by bioturbation or surface water flow. Finally, pre-fertilization data on bulk soil and plant (above and below) N pools and $\delta^{15}N$ signatures were collected (Table 1) and showed no significant difference between plots before nutrient addition treatments when tested for differences using a fixed-effects model with component type (foliar, root, or soil), habitat (i.e., under tree canopy and in open pasture), and future plot designation to explain variation in N content (mg/g). We therefore feel confident that with this experimental design, the sampling plots represent independent measurements of the desired ecosystem components, despite their nontraditional spatial layout.

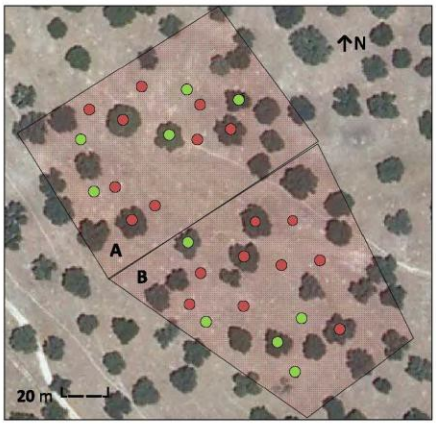

Fig. 1. Aerial view of the experimental setup in the dehesa near Majadas de Tiétar, area A received the N addition treatment, and area B received the N + P addition treatment. Green points are sampling plots that did not receive any ^{15}N label, and red points are labeled plots. Points over trees represent two distinct 4 × 4 m sampling plots, one on either side of the trunk oriented east–west. Points over open pasture represent only one distinct 4 × 4 m sampling plot. N addition was 50 kg N/ha, with an additional 25 kg P/ha in the N + P treatment.

Sampling and sample processing

Plots were sampled five times: one day, seven weeks, seven months, and one year after label was applied, as well as one pre-label sampling which took place one day before fertilization. Sampling took place on 20 March 2017, 22 March 2017, 10–12 May 2017, 19 October 2017, and 21 March 2018, respectively. Samples in May were collected over 3 d with control plot samples being collected the first day, half of the treatment plots sampled the next (evenly distributed across treatments and habitats), and the second half being collected on the final day. This was done due to difficulty in collecting cores with the

Table 1. Summary of pretreatment (19 March 2017) N concentrations by habitat of plant material and surface soil (0–5 cm).

Habitat	Future treatment	Foliar	Root	Soil
Open pasture	N plots	7.68 ± 2.0	9.82 ± 1.5	0.95 ± 0.08
	N + P plots	6.08 ± 1.0	9.80 ± 1.0	1.03 ± 0.05
Under canopy	N plots	18.1 ± 1.8	13.2 ± 1.2	2.62 ± 0.4
	N + P plots	19.5 ± 1.8	13.2 ± 0.9	2.92 ± 0.5

Notes: Values are mg N/g, mean ± standard error, $n = 6$. There were no significant differences between plots prior to nutrient additions.

equipment available and to accommodate laboratory space for processing multiple experimental sets at one time. For each sampling, one 0–5 cm soil core with a 5.5 cm diameter was collected from a random location within each plot, avoiding sampling directly next to edges and previously sampled locations as needed.

The intact cores were placed in a cooler and taken directly to the laboratory (University of Extremadura, Plasencia, ~40 min transfer time) where the aboveground plant biomass was separated, washed, and placed in a drying oven at 45°C for 48 h. The soil portion was sieved through a 2-mm sieve, and the coarse fragment (<1%) removed. All root material was removed, washed, and placed in a drying oven at 45°C for 48 h. Sieved soil was stored field-moist at 4°C overnight. The following day soils were subsampled as follows to determine extractable and chloroform-labile extractable N content (hereafter referred to as microbial biomass N as a more ecologically intuitive term, discussed further below). One 40 g subsample went into 200 mL of 0.5 mol/L K_2SO_4 which was shaken for one h, and then, the supernatant was filtered through Whatman #1 filter paper and frozen. A second 40 g subsample was placed in a glass beaker and fumigated with chloroform in the dark for 72 h and then extracted in 0.5 mol/L K_2SO_4 as above. A final 10 g subsample was taken to determine gravimetric water content and for later stable isotope analysis. All exact weights were recorded for later mass calculations. Frozen extracts and dry soil and plant materials were weighed and then shipped to the Max Planck Institute for Biogeochemistry in Jena, Germany, for further processing. There, dry plant samples were picked through to remove litter and small stones. All dry samples were ground to a fine powder using a ball mill. Subsamples of plant and soil powder were weighed out for ^{15}N, N, and C content analysis.

Chemical analysis of C, N, and ^{15}N

Dry soil and plant samples were run on a Delta-Plus isotope ratio mass spectrometer (Thermo Fisher, Bremen, Germany) coupled via a ConFlow III open split to an elemental analyzer (Carlo Erba 1100 CE analyzer; Thermo Fisher Scientific, Rodano, Italy) to measure their total N and ^{15}N content. Standard deviation of the measured standards was 0.2‰ or better. Soil and plant tissue C concentrations were determined on a Vario EL II (Elementar Analysensysteme GmbH, Hanau, Germany). Extracts from fumigated and unfumigated soils were run on a TN-100 (A1 Envirotech, Düsseldorf, Germany) to determine total extractable N content. Microbial biomass N was calculated as the difference in total extractable N content in fumigated and unfumigated samples (Brookes et al. 1985). These values were not adjusted using any k factor because all comparisons using these values are internally referenced. The remaining volume of extracts was frozen again and shipped to the Stable Isotope Laboratory at Utah State University in Logan, UT, USA, for measurement of ^{15}N content. There, samples were persulfate-digested (Cabrera and Beare 1993) and the digest solution was diffused using the diffusion technique of Stark and Hart (1996). The filter paper products of the diffusion were run on a Europa 20-20 IRMS (Sercon Limited, Crewe, UK) to measure their ^{15}N content with relative deviation of the standards equal to 0.05‰ or better. Due to shipping the extracts multiple times, 12 of the 96 samples for microbial biomass ^{15}N recovery were not available for final ^{15}N analysis (Appendix S1: Table S1).

Label recovery calculations

For each ecosystem component investigated, ^{15}N excess (atom percent excess) was calculated by taking the absolute content of ^{15}N in the sample (atom percent enrichment) minus the natural abundance of ^{15}N found in the same component from unlabeled plots in the same sampling campaign. Recovery for this pool was then calculated as the total amount of excess ^{15}N calculated at the plot level (atom percent excess multiplied by N pool size) divided by the known quantity of ^{15}N that was added to the plot. The exceptions to this were the soil total extractable N and microbial N pools, which were calculated relative to the total ^{15}N excess recovered in the soil of the same plot. Ambient $\delta^{15}N$ of pretreatment (fertilization and labeling) plants and soils were calculated as follows:

$$\delta^{15}N = \left(\left(R_{sample} - R_{standard}\right)/R_{standard}\right) \times 1000$$

where R is the ratio of ^{15}N to ^{14}N in the sample (R_{sample}), and the standard is the ratio in atmospheric air ($R_{standard}$ = 0.3677% ^{15}N).

Statistical analysis

Data were analyzed in R Studio using the R version 1.3.2 (R Core Team 2019), using a split-plot design with nutrient addition treatment as the whole-plot factor and habitat nested within whole plots. We used a mixed-effects model with treatment (N only and N + P), habitat, time, and their interactions as fixed effects and plot as a random effect. As we sampled a relatively short time series (four campaigns post-labeling) over a pronounced seasonal cycle and collected new soil cores, avoiding previously sampled locations within each plot, we treated campaign as a fixed factor without a time series autocorrelation term in the models. Data were tested for normality using visual inspection of histograms and q–q plots, and then transformed when needed by applying Tukey's ladder of powers to transform the original non-normal distribution into one that maximizes the Shapiro–Wilk W statistic. This was accomplished using the function transformTukey() from the package rcompanion (Mangiafico 2019). If the function was unable to produce an optimum transformation, the log or square-root transformation was used as appropriate. Analysis of variance (ANOVA) was then computed using the function lmer() from the package lmerTest (Kuznetsova et al. 2017), and Tukey's honest significant difference post hoc comparisons on estimated marginal means were carried out using the function emmeans() from a package by the same name (Lenth 2016, 2019). Degrees of freedom were calculated using Satterthwaite's method for ANOVAs and the Kenward-Roger method for Tukey tests. Effects and comparisons were considered statistically significant at $P \leq 0.05$. Our original data and example code can be found in the supplementary material (Data S1).

Results

Ecosystem N

The N concentration (mg N/g) of foliar material varied with time and habitat ($P < 0.05$, campaign × habitat, Table 2). The herbaceous layer underneath tree canopies had higher N concentrations in all sampling campaigns, but the magnitude was greatest in March 2017. Foliage had significantly more N in March 2018 than in March 2017 ($P < 0.001$, campaign, Table 2). There was also a decrease in foliar N concentrations in the fall, which statistically differed from the two early-spring campaigns for samples underneath canopies. Nutrient additions resulted in different foliar N concentrations when averaged over sampling campaigns, although the effect was only near significance ($P = 0.051$, treatment). Foliage from plots where only N was added had 1.5–3 mg N/g more N on average than in N + P plots throughout the four sampling campaigns.

Root N concentration followed roughly the same pattern as the aboveground plant pool, but the values were generally less variable. Notably, root N was not higher in March 2018 than in March 2017, as was seen in plant foliage. However, root N concentration did vary significantly across time ($P < 0.001$, campaign, Table 2) with the highest root N concentrations measured in March 2017 and the lowest in October 2017. Root N was significantly higher underneath tree canopies than in open pasture ($P < 0.001$, habitat, Table 2). There was significantly more N in roots when N alone was added, with on average 1–2 mg N/g more than roots in N + P plots throughout the four sampling campaigns ($P < 0.05$, treatment).

Plant tissue C:N did not differ by habitat or nutrient addition treatment, but did differ significantly between plant roots and foliage, and both the magnitude and direction of the difference varied over time ($P < 0.001$, type × time; Appendix S1: Fig. S1). The highest foliar C:N was 36 ± 3 in October 2017, which corresponded to the lowest root C:N of 23 ± 2 (averaged over nutrient addition treatment and habitat). Soil C:N was unaffected by nutrient addition treatments or time, but did vary by habitat ($P < 0.001$, habitat; Appendix S1: Fig. S2). Under-canopy soil had higher C:N (13 ± 0.1) than open pasture soil (11 ± 0.1), mostly due to higher absolute C concentration under canopies (44 ± 4 vs. 12 ± 1 mg C/g soil for under canopy and open pasture, respectively).

Soil N concentration was much higher underneath canopies than in open pasture ($P < 0.001$, habitat, Table 2). There was a significant interaction between treatment and time ($P < 0.01$), in spring 2017 (for the first two sampling campaigns), there was more N in soil from the N-only plots, while in March 2018 there was

Table 2. Nitrogen content of measured ecosystem components, values represent mean ± standard error with combined nutrient treatments, $n = 12$, except for foliar samples for 19.10.2017 where only 7 and 11 samples had sufficient plant material for processing in open grassland and under-canopy plots, respectively.

Habitat	Campaign	Foliar (mg N/g)	Root (mg N/g)	Soil (mg N/g)	Total E (μg N/g) soil	Microbial (μg N/g) soil
Open pasture	March 2017	9.46 ± 0.8 a	10.32 ± 0.7 a	1.26 ± 0.1 a	43.9 ± 10.2 a	52.10 ± 5.0 ab
	May 2017*	10.68 ± 1.4 a	8.77 ± 0.6 ab	1.10 ± 0.1 ab	14.83 ± 1.5 b	42.19 ± 2.6 ab
	October 2017	8.08 ± 1.1 a	6.19 ± 0.8 a	1.33 ± 0.2 a	33.61 ± 3.8 a	54.97 ± 4.0 a
	March 2018	22.15 ± 1.8 b	7.31 ± 0.9 b	0.87 ± 0.1 b	14.13 ± 1.3 b	39.13 ± 1.8 b
Under canopy	March 2017	19.22 ± 1.8 a	12.64 ± 0.8 a	3.41 ± 0.2 a	128.75 ± 28.6 a	117.19 ± 14.4 a
	May 2017*	15.20 ± 0.6 ab	9.77 ± 0.8 b	3.60 ± 0.5 ab	55.34 ± 10.9 ab	112.59 ± 16.7 a
	October 2017	11.75 ± 0.7 b	9.06 ± 0.7 b	3.03 ± 0.3 ab	37.62 ± 5.0 b	110.19 ± 10.5 a
	March 2018	28.12 ± 1.9 c	10.95 ± 0.6 ab	2.55 ± 0.2 b	24.22 ± 2.6 b	107.49 ± 9.6 a

Notes: Root and soil samples are from 0 to 5 cm depth. Letters represent Tukey post hoc groupings within a single spatial location and ecosystem component.

*Half of the samples (distributed evenly across treatments) were collected on 21 May 2017, the second half on 22 May 2017.

significantly more N in the N + P plots (2.0 ± 0.3 vs. 1.4 ± 0.2 mg N/g soil in March 2018 for N + P and N only, respectively). Overall, the lowest soil N pools were measured in March 2018.

Total extractable soil N was generally higher under canopies ($P < 0.001$, habitat, Table 2). Time affected the total extractable N of the two habitats differently. This pool consistently decreased across the sampling campaigns underneath canopies but oscillated in the open pasture soil ($P < 0.01$, habitat × campaign, Table 2). Total extractable N was not affected by the nutrient addition treatments.

Microbial biomass N (chloroform-labile N) was most strongly affected by habitat, with much larger microbial N pools under tree canopies ($P < 0.001$, habitat, Table 2). There was a significant interaction between the nutrient addition treatments and sampling campaign. This was driven by the final sampling campaign in March 2018 where the N + P plots averaged 86 ± 9 μg microbial N/g soil and the N-only plots averaged 61 ± 14 μg microbial N/g soil ($P < 0.05$, averaged over habitat). Degrees of freedom, F statistics, and *P*-values for all ecosystem N pools are available in Appendix S1: Table S2.

^{15}N label recovery

Label recovery in both foliar and root pools was greatest in March 2017, the first sampling campaign after label application ($P < 0.05$, time, Table 3). In foliage, there was consistently greater recovery in open pasture plots than under-canopy plots ($P < 0.001$, habitat, Table 3). Label recovery in roots had a slightly different trend, with consistently higher recovery in open pasture plots in the first three sampling campaigns but the opposite in March 2018 ($P < 0.001$, habitat × time, Table 3). There was no significant effect of the nutrient addition treatments on label recovery in foliage or roots.

Label recovered in the bulk soil was nearly significantly affected ($P = 0.057$) by the three-way interaction of habitat, treatment, and time (Fig. 2). This is because time significantly influenced recovery in open pasture samples from the N addition plots, but not open pasture samples from N + P plots nor any samples from under-canopy soils. This carried over into a significant habitat by time interaction ($P < 0.01$) and a significant effect of time on its own ($P < 0.01$). Soil label recovery was greater in October than in May 2017 or March 2018 (Table 3).

Percent of label recovered in soil pools was calculated relative to recovery in soil from which that sample was extracted, because any label found in soil N pools would by definition also be part of the label recovered in the bulk soil. There was a significant interaction between habitat and time for label recovered in the total extractable N pool. Overall, there was more label recovered in the total extractable N pool underneath canopies (22% ± 4% on average across treatments and time, Fig. 3) compared to open pasture (14% ± 2%, $P < 0.05$). For open pasture samples, the most label was recovered in this pool in

Table 3. Percent of added ^{15}N recovered in main ecosystem components, values represent median ± standard error with combined nutrient treatments, $n = 12$, except for foliar samples for 19 October 2017 where only 7 and 11 samples had sufficient plant material for processing in open grassland and under-canopy plots, respectively.

Habitat	Campaign	Time since label application	Foliar	Root	Soil	Sum
Open pasture	March 2017	24 h	13.2 ± 2.5	7.2 ± 1.5	23.0 ± 5.1	41.3 ± 6.7 a
	May 2017†	9 weeks	4.3 ± 2.5	5.4 ± 1.3	18.9 ± 4.0	27.4 ± 6.5 ab
	October 2017	7 months	3.5 ± 1.0	2.9 ± 1.0	47.6 ± 5.2	**62.8 ± 6.2 a**
	March 2018	1 yr	3.1 ± 0.6	1.2 ± 0.6	20.8 ± 3.2	25.0 ± 3.5 b
Under canopy	March 2017	24 h	9.7 ± 1.7	3.8 ± 0.8	29.1 ± 8.5	40.9 ± 9.8 a
	May 2017†	9 weeks	3.0 ± 0.4	2.2 ± 0.5	20.8 ± 2.2	27.8 ± 2.6 b
	October 2017	7 months	1.8 ± 0.3	1.4 ± 0.4	22.9 ± 5.1	**24.7 ± 5.6 b**
	March 2018	1 yr	2.7 ± 0.4	2.5 ± 0.7	20.1 ± 3.4	27.7 ± 3.7 ab

Notes: Root and soil samples are from 0 to 5 cm depth. Final column is the sum of the above ground, root, and soil percent label recovery and represents the total amount of added label recovered in the herbaceous layer down to five cm soil depth in all measured pools. Letters represent Tukey post hoc groupings within a single spatial location. The bold values are a significant vegetative cover difference in label recovery 7 months after application, $P = 0.002$.

† Half of the samples (distributed evenly across treatments) were collected on 21 and the second half on 22 May 2017.

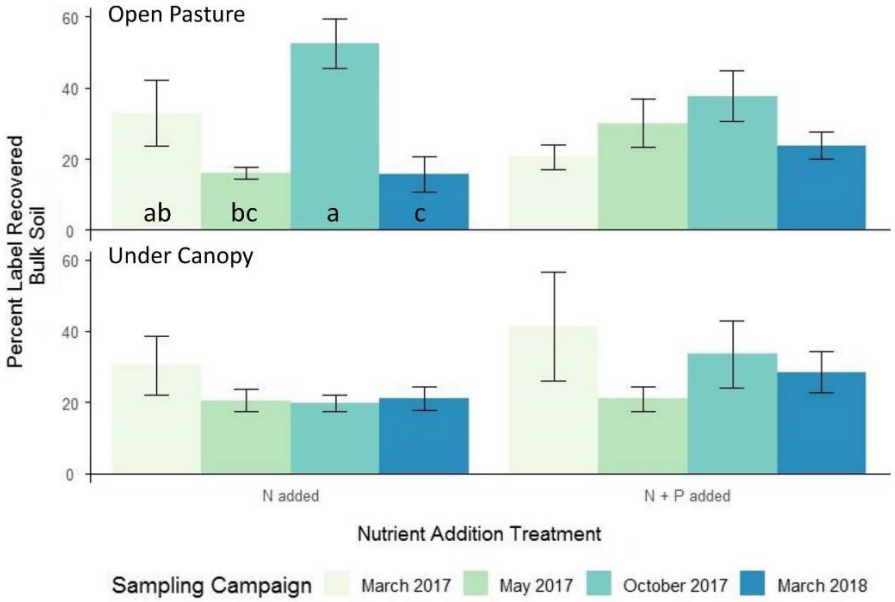

Fig. 2. Label recovery in bulk soil over four sampling campaigns for open pasture (above) and under-canopy (below) plots with two different nutrient additions. Values are mean ± standard error, $n = 6$. Samples are from 0 to 5 cm depth. Letters represent statistically different Tukey's post hoc groupings within the open pasture N addition treatment.

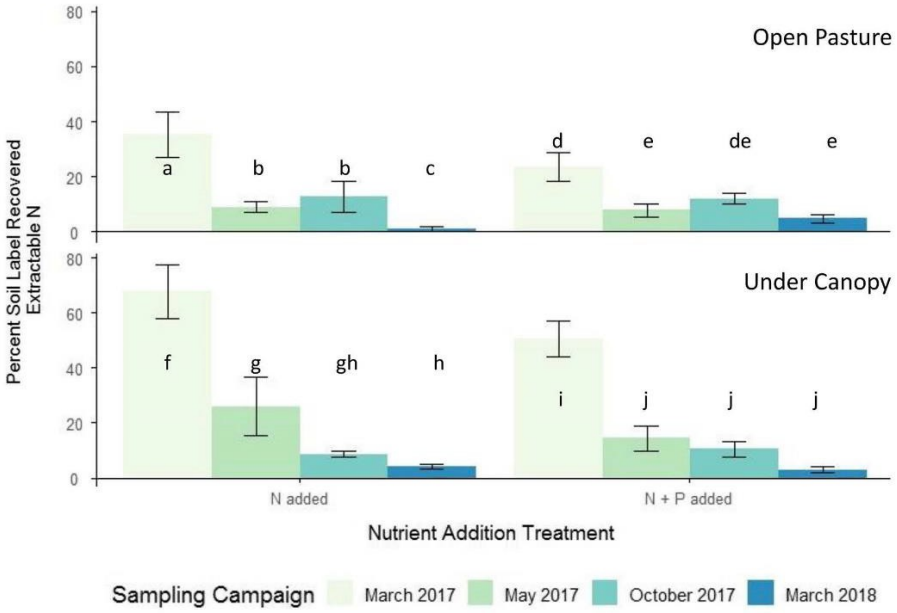

Fig. 3. Label recovery in extractable N over four sampling campaigns for open pasture (above) and under-canopy (below) plots with two different nutrient additions. Values are mean ± standard error, see Appendix S1: Table S1 for sample sizes. Samples are from 0 to 5 cm depth. Letters represent Tukey post hoc groupings within one habitat × nutrient addition combination.

March 2017, statistically more than recovery in any subsequent sampling campaign, decreasing to near the detection limits of the method by March 2018 ($P < 0.05$, habitat × time, Fig. 3). For under-canopy samples, the greatest recovery in total extractable N was also in the first sampling, but because this pool was overall larger, amounts were easily detectable even in March 2018.

Microbial biomass ^{15}N recovery (recovery from the chloroform-labile pool) was significantly influenced by the three-way interaction between habitat, time, and treatment, although marginally so ($P = 0.048$). However, due to the sensitivity of this measurement to sample loss (both the unfumigated and fumigated samples have to be carried successfully through to the end of the diffusion procedure), some habitats by nutrient addition time steps were only represented by a few samples (Appendix S1: Table S1). Therefore, we focus on results of the lower order ANOVA effects (single factors and two-factor interactions). Unlike other soil pools, label recovery in microbial biomass N was significantly affected by habitat and treatment ($P < 0.05$, for the interaction, Fig. 4). This is because microbial biomass recovery was greater in open pasture when N alone was added, but there was no treatment effect under trees. In general, the label recovered in microbial biomass decreased with time, from 24% ± 9% in March 2017 to 10.8% ± 2% in March 2018 (averaged across nutrient addition treatments and habitat).

Total ^{15}N label recovery

Total label recovery was calculated as the sum of label recovered in foliage, roots, and soil. Overall recovery was affected by the interaction

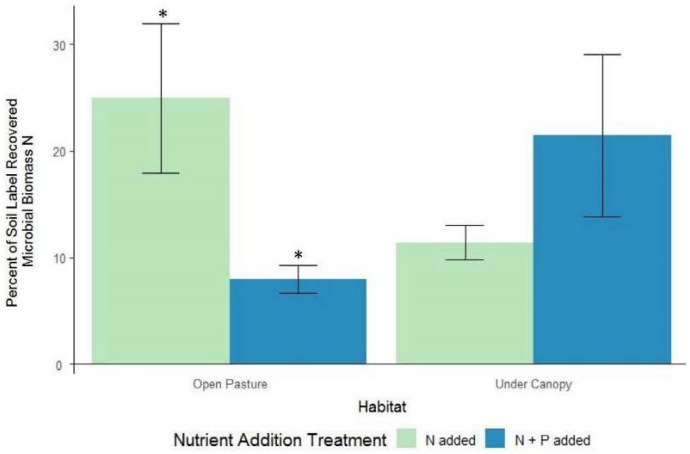

Fig. 4. Label recovery in microbial biomass for two habitats under two different nutrient addition treatments. Values are mean ± standard error over all sampling campaigns, n = 12–18 (see Appendix S1: Table S1 for details).

between habitat and time ($P < 0.01$, Table 3). Within one habitat type, open pasture plots had the greatest label recovery in October 2017, but below-canopy plots had the greatest recovery immediately after label application in March 2017. Comparing between habitats, open pasture had significantly greater label recovery than under canopy, and this effect was strongest in October 2017 (Table 3). The overall mean of open pasture recovery was 40.5%, compared to 34.9% for under canopy (averaging across sampling campaigns). Maximum recovery in plots where N alone was added was 54% ± 7% in March 2017, and the minimum recovery was 24 ± 4% in March 2018. In comparison, maximum recovery in N − P plots was 46 ± 9% in March 2017 with a minimum of 33% ± 4% in March 2018. Degrees of freedom, F statistics, and P-values for all ^{15}N label recovery variables are available in Appendix S1: Table S3.

Discussion

Total ^{15}N recovery in this study

We set out to determine the effects of habitat, P availability, and time on the fate of N additions in a dehesa using a ^{15}N tracer. Compared to other ^{15}N tracer experiments, our total label recovery was relatively low, around 40% one day after onset, while typical grassland tracer experiments recover 52% in the short term (one week to one month, Templer et al. 2012). There are four potential reasons for this: initial sample processing, grazing, leaching, and unmeasured pools. In March 2017, immediately after label application, foliar samples were rinsed with distilled water before further processing. This step, while critical to accurately measuring the true ^{15}N content of the plant material, likely removed residual label on the surface of the foliage. We minimized this effect by adding an additional 0.5 L of water to the plots after label application in the field, but this volume may have been insufficient. This label loss is only relevant to the first sampling campaign, because rain showers at the end of March 2017 (Appendix S1: Fig. S3) would have washed residual label off the foliage surface.

Between March and May 2017, we believe the primary loss of label was through grazing. Estimated biomass removal from grazing, based on exclusion cages within the same dehesa, is 50 kg

dry matter·ha^{-1}·month^{-1} during the spring. Assuming 1% N content of the vegetation and an atom percent excess of 0.14 (grand average for foliage in this study; Appendix S1: Table S4), 28 mg $^{15}N/m^2$ would have been removed by cattle between March and May 2017, 54% of our added label. This estimate is conservative as atom percent excess of foliage in March 2017 was 0.37 for open pasture and 0.20 for under canopy and measures of material removed by grazing in 2017 for other fertilized sites in the area were 120 kg dry matter·ha^{-1}·mo^{-1} (G. Moreno, *personal observation*, but see also ancillary information reported for sites ES-LM1 and ES-LM2 in the European Fluxes Database Cluster). However, it must be noted that some label was likely returned to plots as urine and dung, and we do not have grazing estimates for the experimental area itself, only sites nearby.

Leaching also likely accounts for some label loss, especially given our shallow (0–5 cm) sampling depth. Due to seasonal rainfall, leaching most likely occurred from March to May 2017 and October 2017 to March 2018 (Appendix S1: Fig. S3). If soil rewetting happens before plants and microbes are physiologically active in the fall, tracer present in the form of nitrate (NO_3^-) would have readily transferred below five cm. Indeed, in Mediterranean systems, rainfall with dry prior conditions leads to increased NO_3^- in local watersheds due to accumulated soil NO_3^- flushed from pore spaces (Bernal et al. 2005, Llorens et al. 2011).

Finally, one potential unmeasured pool is litter (standing and flattened dead material from the herbaceous layer, including tree litter under canopies). When sampling vegetation, browned and browning components were intentionally discarded. However, given our data we believe the role of litter as an N sink in this system is not insignificant (discussed in *Fate of added ^{15}N*). This oversight was due to the low ground cover of litter at the site (3% cover in May 2017, data not shown). In other studies, litter accounted for 25.5% of grassland tracer recovery (Templer et al. 2012); however, this is likely higher than our site, given the low litter cover in dehesas (Casals et al. 2010). A second unmeasured pool is label taken up by oak trees. However, Holm oak trees do not respond readily to fertilization even after two years (Rivest et al. 2011); therefore, this ecosystem component is likely a small sink for ^{15}N.

Despite the factors contributing to low recovery, this phenomenon is not unique to this experiment. Hart et al. (1993) had 44% total recovery one year after application to forest soil. Nadelhoffer et al. (1999) was similar with 45.5% total recovery in an oak forest, despite sampling to 20 cm soil depth. Mauritz et al. (2014) found just 10–12% recovery in annual plants of a semiarid chaparral system one year after label application. These studies cite various reasoning for their low label recovery such as dry conditions at onset of label application (Hart et al. 1993), horizontal mixing with natural abundance material diluting a relatively low-level tracer (Nadelhoffer et al. 1999), and sample processing error (Mauritz et al. 2014), the first and the last of which are similar to contributing factors in our study. All of these examples are systems ungrazed by livestock. It is also known that there is a negative correlation between the abundance of fine roots and tracer recovery (Templer et al. 2012) and fine roots are especially abundant in our site within the surface soil (Moreno et al. 2005, Rolo and Moreno 2012, Nair et al. 2019).

Ecosystem N pools

There was a seasonal pattern in N concentrations of bulk surface soil and plants, which declined in each sampling campaign throughout the year following the first growing period. At the peak of the second growing period, in March 2018, N concentration in plants increased (dramatically in foliage, Table 2). At the same time, there was a slight decrease in soil N and a very noticeable decrease in extractable soil N pools which indicate that plant N uptake was great enough to reduce other surface N pools. Likely, the increase in plant N is a result of interannual variability rather than a delayed fertilization effect as the growing period of 2017 was characterized by low productivity due to low precipitation during the spring and summer periods (Luo et al. 2018) with rainfall only 60% of the annual average (data not shown, but see Appendix S1: Fig. S3). The timing and magnitude of the dehesa springtime productivity peak are extremely sensitive to rainfall and temperature conditions of that year (Luo et al. 2018). Because soil was already relatively dry during fertilization in

2017, it is likely that plants were not able to fully utilize added nutrients until the following growing season. However, this is not supported by our low tracer recovery in plant pools in March 2018 (foliar N content is high, Table 2, when foliar ^{15}N recovery is low, Table 3), possibly because much of the biologically available tracer had been removed from the system due to grazing of the initially highly labeled material.

Ecosystem N concentrations were strongly affected by habitat with all pools having on average higher N concentrations underneath tree canopies. Surface soil N and microbial biomass N were between two and three times greater under tree canopies. This is as expected as dehesa trees create islands of fertility with enhanced nutrient content and higher microbial biomass (Dahlgren et al. 1997, Lopez-Sangil et al. 2011), despite a slightly higher C:N ratio of the under-canopy soil, likely due to higher C:N ratio of tree-derived litter (Cardinael et al. 2018). As our site has relatively low tree density (<20% tree cover), in general effects seen in the open pasture areas would be more representative of overall ecosystem behavior.

For plant tissues, addition of N alone resulted in slightly higher N concentrations than N + P addition. This was not the case for microbial biomass, which did not differ between nutrient addition treatments except for the last sampling campaign where the N + P treatment had higher biomass N. The treatment effect in plant tissues is possibly a result of the form of fertilizer applied, which was dominated to a greater extent by NO_3^- in the N-only plots. NO_3^- is more soluble than NH_4^+ and may have been more readily taken up by plants during the relatively dry 2017 growing season. Alternatively, it is possible that the many different species that make up the dehesa herbaceous layer on average prefer NO_3^- (Britto and Kronzucker 2013). Previous work has found that dehesa pasture vegetation is nutrient-limited, but responses are easily confounded by other limiting factors such as light or water limitation (Moreno 2008). Generally, plant biomass is the metric used to gauge nutrient limitation in response to fertilization, but this is not possible for this study because of active grazing. It is known that the herbaceous layer of the Majadas dehesa is more productive with N addition and shows an additional increase with N + P fertilization (Perez-Priego et al. 2015, Migliavacca et al. 2017).

The greater microbial biomass N found in N + P plots in March 2018 indicates that either microbe was able to take up more N in the presence of P or there was greater microbial biomass when N and P were added, but that the effect was not immediate. However, because the label recovery in microbial biomass did not follow the same pattern, we cannot be sure where this increased biomass N came from. Because plant root tissue also increased in N concentration in March 2018, the source may be higher N rhizodeposition, but one would have expected to see this pattern in both treatments. Because we only see it in microbes in the N + P treatment, perhaps it is a result of a subtle change in plant rhizosphere chemistry (Dijkstra et al. 2011) in the presence of added P. Alternatively, it could be a shift in plant community composition and corresponding rhizosphere chemistry due to N + P addition vs. N-only addition over the year (Ochoa-Hueso et al. 2013).

Fate of added ^{15}N

We hypothesized that open pasture areas would retain more added N due to their lower SOM and presumed greater N limitation compared to under-canopy areas (H1). The label recovery in plants was significantly higher in the open pasture plots for most sampling campaigns (Table 3). This supports the idea that more N was retained in this lower nutrient availability environment, but the magnitude of the difference is small compared to that seen in the soil data. While the overall mean (combining nutrient addition treatments) for total label recovery was greater in open pasture plots, this effect was driven by soil label recovery in October 2017 (Table 3).

The peak in soil ^{15}N recovery seen in October 2017 may relate to a peak in decomposition and degradation of the herbaceous layer litter throughout the intensely irradiative summer. The litter layer would have been composed almost entirely of recently senesced, labeled foliage from the previous growing season. Physical fragmentation and photodegradation control litter degradation rates in semiarid systems (Coûteaux et al. 1995, Austin and Vivanco 2006), and label stored in this pool would have reached

its maximum transfer to the soil pool by October 2017. Additionally, direct spraying is not the only way label can get into the litter pool. Initial decomposition involves an increase in litter N content (Parton et al. 2007) from investment of exoenzymes (which have high N content). This was likely greater for N-only plots because of increased microbial demand for P, which could also be obtained from litter. Therefore, we believe that low soil label recovery of N-only plots compared to N + P plots in May 2017 (Fig. 2) is due to greater litter N immobilization in the absence of increased P availability. Somewhat surprisingly, in Parton et al. (2007), arid grassland litter was the only litter type which did not show a pattern of initial N investment. However, they only tested one litter sample from one arid grassland. Our results suggest that the pattern may hold at least for some semiarid grassland litters. In total, these data imply that there is more complete biomass turnover throughout the year in open pasture areas of dehesas, which might be enhanced in the presence of greater N availability.

We also hypothesized that increased P availability would prevent P limitation and enhance N retention in plant and microbial pools relative to addition of N alone (H2). We found little evidence for co-limitation, given that there was no consistently greater recovery in N + P plots compared to N-only plots. We did find that there was greater label recovery in open pasture microbial biomass of N-only plots (Fig. 4). We interpret this as slower growth and lower turnover of the microbial biomass pool in open pasture plots when N and P are imbalanced. This is supported by statistically lower microbial biomass N concentrations in the N-only plots compared to N + P plots in March 2018, indicating that potentially microbes in N-only plots were growing slower. Reduced growth is a fairly common response of soil microbes to N addition (Treseder 2008, Riggs and Hobbie 2016). A broad-scale assessment of grassland microbes found that the addition of N alone affected the microbial community differentially than N + P addition (Leff et al. 2015), which could lead to differential turnover. Different SOM concentrations and chemical compositions underneath tree canopies also support a distinct microbial community (Ho et al. 2017), which did not show this differential response to stoichiometric imbalance. However, because the nutrient addition treatments may have affected communities in both habitats, it is not possible to say what role this played. Overall, low label recovery in microbial biomass when N and P were added was surprising given that previous research done at the site indicated that soil microbes were co-limited by N and P (Weiner et al. 2018).

With regard to the effect of time (H3), we expected to find that added N would first be taken up in microbial biomass (Kuzyakov and Xu 2013), then move into the extractable soil N pool as a result of inorganic N accumulation (Austin et al. 2004), and end mostly in the plant pool one year later (Kuzyakov and Xu 2013). Contrary to our expectation, the highest plant recovery was in March 2017, just 24 h after label was applied. High recovery immediately after application suggests that much of the label was taken up through leaf tissue (Sparks 2009, Nair et al. 2016), meaning that plants were never in competition with microbes for this N. The slight increase of label recovery in plant tissues under tree canopies from fall 2017 to spring 2018 could indicate the use of N leached to soil below five cm.

Regardless of season, the largest individual sink for added N was the soil. This is consistent with previous tracer studies in grasslands for weeks to months after addition (Templer et al. 2012). However, none of the studies in the 2012 review deal directly with seasonal effects, which played an important role in our study, because we found the highest soil label recovery in the fall. A study similar to ours, which addresses temporal effects on the fate of N additions in Mediterranean shrublands, also found a peak in N recovery within the soil inorganic N pool in the fall (Dias et al. 2012) and attribute it to litter decomposition. This fall peak was only found in plots with added N, not in unfertilized plots, with a rate of N application very similar to ours (their study had 40 or 80 kg N/ha, ours 50 kg N/ha). We did not measure inorganic N pools, but it is likely that the peak in total extractable N (inorganic N plus organic N) concentrations seen in October 2017 is due to increased inorganic N, which generally increases in arid and semiarid soils throughout the dry season (Gallardo et al. 2000, Austin et al. 2004). Given this pattern, if the onset of autumn rain is acute, N would be susceptible to loss from the system via leaching

(Bernal et al. 2005, Llorens et al. 2011, Dijkstra et al. 2012). However, our label recovery in total extractable N and microbial N does not follow the same pattern (see Table 3, Fig. 3) so we cannot rule out the possibility that high soil label recovery in the fall is purely from physical breakup of labeled litter, with mineralization playing little to no role. Additional experiments would be needed to make this distinction.

In a Californian Mediterranean grassland, Hart et al. (1993) had relatively high label retention (33%) in non-extractable soil N, which they attributed to abiotic fixation by vermiculite and SOM. Vermiculite is not present at our site, but smectite is (Pérez Arias 1992, Muñoz et al. 1995), and likely contributed to soil ^{15}N retention in addition to SOM. This is evident by the fact that only 2.5–7.5% of total soil N was extractable (including chloroform-labile N). In other words, as much as 97.5% of soil N was not extractable by 0.5 mol/L K_2SO_4, indicative of high abiotic N fixation. Additionally, the highest recovery of ^{15}N in total extractable and microbial N pools was immediately after label application, indicating little reverse flow of label after abiotic fixation. Previous work comparing biotic vs. abiotic fixation rates found that on average 76% of added NH_4^+-N was abiotically fixed in an N-poor, sandstone-derived soil (Johnson et al. 2000), similar to that at our field site. If the liquid application of labeling solution led to faster or more efficient fixation of ^{15}N, relative to the pelleted fertilizer N, this could explain the low ^{15}N recovery in plant tissues during March 2018 when tissue N concentration was highest. This is because the abiotically fixed ^{15}N, if initially quickly sorbed, would have been less available for biological uptake throughout the remainder of the experiment.

Conclusion

We found no strong effect of increased P availability on the fate of N additions; rather, we found generally equal N allocation between the two nutrient addition treatments. However, one should bear in mind that 2017 was a dry and low productivity year. More work should be done to determine how water availability might influence N allocation and potential N:P imbalances in dehesas and similar ecosystems. We show that habitat plays a fundamental role in how N is cycled in this system. However, although soil N content was up to three times higher beneath canopies, this barely affected the fate of N during the spring growing season when most grass biomass is produced. Our major finding is that soil retained most of the added N, regardless of time since application, P availability, or habitat. If excess N is mostly abiotically fixed in soil, as our results imply for a dry year, moderate rates of N deposition may not have much impact on ecosystem function because the majority of added N would not be biologically available. The strong seasonal effect we found in open pasture plots (especially with only N added) implies that the timing of rainfall onset relative to the onset of plant growth will be critical to ecosystem N retention in the future. Additional work is needed to determine whether these findings are generalizable to wetter years and therefore how this phenomenon could feedback into interannual ecosystem C dynamics.

Acknowledgments

The authors thank the Alexander von Humboldt Stiftung for financial support of the MaNiP project through the Max Planck Research Prize to Markus Reichstein. Thanks also go to Kirsten Küsel, Markus Reichstein, and Susan Trumbore for stimulating discussion of the data. Finally, the authors thank Yonatan Cáceres Escudero, Heike Geilmann, Jinhong Guan, Tiana W. Hammer, Iris Kuhlman, Mónica Lorente, Adrian Mateos, and Rolf Rödger for tireless support in the field and in the laboratory. The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2921/full

Chapter 4

Microbial carbon-use efficiency is not limited by the availability of nitrogen and phosphorus

Abstract

The extent to which soil microbes are limited by the availability of nutrients such as nitrogen (N) and phosphorus (P) is poorly understood, especially in regards to how fertilization changes microbial carbon-use efficiency (CUE). CUE is the fraction of C converted into biomass out of all C taken in and plays a critical role in global C budgets. Using the ^{18}O labeled water method we tested short vs long-term effects of N and/or P fertilization on microbial CUE and C, N, and P-acquiring enzyme activities in two soils from an oak-savanna which differ in their SOM content. We hypothesized that soils with more SOM (from under tree canopies) would have greater CUE than soils with less SOM (from open grassland), that CUE would increase with fertilization and that this increase would be associated with a decrease in enzyme activity in the short term, but a change in community and increased extractable C (resulting from increased plant productivity) in the long term. We found that nutrient additions did not affect microbial CUE in the relatively high SOM habitat on either time scale. In contrast, the low SOM habitat had lower CUE when single nutrients were added, with significantly reduced CUE when P alone was added, but was unchanged when N and P were added together. Our results indicate that in the short-term, stoichiometric imbalances can reduce microbial CUE, but further study is needed to determine how long such effects would last. Overall our results show that CUE is not nutrient limited, rather it is limited by the amount of C available to soil microbes.

Introduction

Microbial carbon-use efficiency (CUE), or the fraction of carbon (C) that microbes put into biomass out of all that they take in, is a key factor in terrestrial C cycling because it controls the amount of decomposed C which will stay in the soil system as microbial biomass. Modelled and experimental calculations of CUE fall between 0.2 and 0.8, with most values around or below 0.4 (Manzoni et al., 2012; Lee and Schmidt, 2014; Sinsabaugh et al., 2016). CUE has been extensively studied using ^{13}C tracers as substrates for microbial growth, but it is uncertain how representative such studies are of actual soil conditions, namely because tracers limit the scope of data interpretation to the case of a specific labelled substrate (Herron et al., 2009; Sinsabaugh et al., 2013; Geyer et al., 2019). This decreases our ability to

determine effects of *in situ* conditions with more complex substrate availability, and where other environmental drivers, such as nutrient availability, could influence CUE. The recent development of a method based on incorporation of ^{18}O labeled water into microbial DNA as a proxy for microbial growth is extremely promising, because it is substrate non-specific (Spohn et al., 2016a; Geyer et al., 2019; Pold et al., 2019). Because microbial CUE is a critical value in recent globally-applied microbial-explicit soil C models (Hagerty et al., 2018; Woolf and Lehmann, 2019), better understanding is needed as to how it varies under different environmental conditions.

An aspect of CUE that is especially poorly understood is under what conditions it is sensitive to changes in nutrient availability (Manzoni et al., 2012; Spohn et al., 2016b; Poeplau et al., 2019). It has long been hypothesized that a trade-off exists in microbial physiology between production of nutrient-acquiring enzymes (such as N-acetylglucosaminidase (NAG) and phosphatase) and biomass, because both require C and energy to build (ignoring for the moment other potentially limiting factors such as moisture or temperature). Therefore, more C and energy resources (in the form of ATP) would be put into building biomass if fewer enzymes were needed to meet nutrient demands, resulting in increased growth and higher CUE with nutrient addition (Schimel and Weintraub, 2003; Manzoni et al., 2012; Riggs and Hobbie, 2016; Spohn et al., 2016b). However, it is also possible that microbes are not nutrient limited, but are instead purely C limited, in which case addition of mineral nutrients would not affect growth or CUE. Support for this was found in previous studies of CUE (Elliott et al., 1983; Riggs and Hobbie, 2016), and in studies of related measures such as microbial respiration and metabolism (Hobbie and Hobbie, 2013; Poeplau et al., 2016; Soong et al., 2018). Likely both hypotheses are valid, with individual ecosystems falling somewhere along a C to nutrient limited spectrum.

One difficulty in addressing the topic of nutrient limitation is that it is often confounded with the effect of time, specifically time since nutrient addition. A primary tool for assessing nutrient effects on emergent ecosystem processes, such as CUE, is fertilization. However, fertilization can also result in changes in plant and/or microbial communities (Gough et al., 2000; del Mar Alguacil et al., 2010; Ramirez et al., 2012) and downstream changes in soil chemistry as a result (*i.e.*, changes in litter quality, as in Liu et al., 2016). Changes in litter chemistry are known to lead to direct changes in microbial CUE and are associated with changes in microbial substrate use (Manzoni et al., 2012; Sinsabaugh et al., 2016), which should be detectable in the C-isotopic signature of respired C (Andresen et al., 2018). Because downstream effects of fertilization are confounding true fertilization (true nutrient availability) effects in most studies of CUE to date (Riggs and Hobbie, 2016; Spohn et al., 2016b; Poeplau et al., 2019), it is possible that direct or short-term responses of microbial CUE to fertilization differ from previously

reported results. Therefore, to address the question of whether or not microbes are nutrient limited, it is important that short-term fertilization responses are considered separately from long-term responses. By quantifying the microbial community and the isotopic signature of microbial respiration at different time-points, we can enhance our understanding of the mechanisms behind short-term and long-term responses to fertilization.

Beyond temporal effects, there is also the issue of site to site variation in biotic and abiotic attributes which might affect the response of CUE to nutrient additions. Between-site comparisons across larger geographic regions integrate effects of differences in climate, parent material, vegetation cover or land management, thus hampering the attribution of observed differences to specific drivers. The variation of potential drivers can be reduced by studying ecosystems where content and composition of SOM varies spatially within the same site and native soil material, as is found in oak-savannas. In these tree-grass ecosystems, oak trees create islands of soil enriched in SOM compared to the surrounding grassland due to *in situ* decomposition of tree detritus (Gallardo, 2003; Moreno et al., 2007). Soils under tree canopies are well-documented to have larger nutrient pools, and one can assume a correspondingly distinct SOM chemical signature (Quideau et al., 2001; Vancampenhout et al., 2009). Such ecosystems offer a valuable opportunity to test the response of important ecological functions in soils with varying SOM content, while holding many other parameters constant.

In order to determine short vs long term effects of fertilization on microbial CUE and how these responses vary with SOM content, we measured CUE (via the ^{18}O labeled water technique) and related variables (*e.g.*, NAG activity, $\Delta^{14}C$ of respired carbon, microbial stoichiometric ratios), in soil with laboratory-applied fertilizations of nitrogen (N) and/or phosphorus (P), as well as soil from a historically fertilized plot which received N and P, from both open grassland areas and under tree canopies in a Mediterranean oak-savanna. We set out to test the following hypotheses:

1) Microbes in soil under tree canopies where soil is relatively enriched in SOM and thus richer in C and nutrients will have higher CUE than microbes from open grassland areas (relatively low SOM).
2) Microbial CUE will increase in the short-term with nutrient addition due to lower investment in enzymes, with soil that receives both N and P showing the greatest increase.
3) Microbial CUE will increase in the long-term with nutrient addition. This will not be associated with lower investment in enzymes, but rather with changes in substrate use and a shift in microbial communities (H4).
4) Microbial communities will shift in the long-term in response to fertilization, but not the short-term.

Materials and Methods

Soil Collection and Preparation

Soil in this study came from an oak-savanna near Majadas de Tiétar (39°56'25"N 5°46'29"W), which since 2004 has been the site of an eddy covariance tower (European Fluxes Database Cluster, identifier 'ES-Lma'). At this site in 2014 a large-scale fertilization experiment was established within neighboring eddy covariance footprints after a full year of pre-fertilization data was collected to ensure comparability of the plots (El-Madany et al., 2018; Nair et al., 2019). One of these footprints was fertilized with 50 kg P ha^{-1} (triple superphosphate) in winter of 2014 and 100 kg N ha^{-1} (as ammonium nitrate) the following spring. A repeat fertilization took place the following year with 25 kg P ha^{-1} (triple superphosphate) in winter of 2015 and 50 kg N ha^{-1} (as ammonium nitrate) the following spring (Luo et al., 2018). We used this a priori fertilized tower footprint as a source of soil with a historical +N+P addition effect. On February 5th 2018, within the +N+P and main (no fertilization) tower footprints, 30 soil cores (5-cm deep, 5.5 cm diameter) were collected from both under tree canopies and within open grassland areas (60 total). Twelve of these cores came from the historically fertilized plot, evenly distributed between the two habitats (under canopy and open grassland). Unequal numbers of cores were collected because not as much soil was needed for our experimental design from the historically fertilized plot. This soil is an Abruptic Luvisol with 79% sand, 20% silt, and 1% clay in the surface horizon under tree canopies, and 74% sand, 20% silt, and 6% clay in the surface horizon in open areas.

Collected cores were pooled into habitat and original (field-based) nutrient treatment sets and sieved to 2 mm. When not being actively processed, soil was stored at 4°C. After sieving, soil from the two habitats of the main plot (no fertilization) was split into four equal subsets for each habitat type. These four subsets would become the control, N addition, P addition, and +N+P addition treatments for each habitat type, respectively (Figure 1).

Samples were wet-up to 60% of water-holding capacity (WHC) and nutrients were added to the samples from the main plot as outlined in Table 1. These additions are equivalent to the 100 kg N ha^{-1} and 50 kg P ha^{-1} applied at the historically fertilized plot, where appropriate to the treatment type. Nutrient additions were delivered dissolved in the water added to bring samples to 60% WHC via a fine mist with

a manual spray bottle and great care was taken to distribute the solution evenly across the entire volume of soil. No nutrients were added to the control or historically fertilized soils, but the WHC was adjusted. Wet-up soil was thoroughly mixed and divided into four replicates for each unique nutrient addition and habitat combination (n = 4 throughout). Pre-incubation times listed below began immediately after completion of this manipulation.

CUE Measurements

The four replicates per treatment and habitat combination used for determining CUE were pre-incubated for 5 d at 20°C before the onset of measurements. We used the ^{18}O method wherein incorporation of ^{18}O labeled water into DNA is used as a measure of growth in order to avoid substrate-specific biases which derive from ^{13}C based methods. This is described in detail in Spohn et al. (2016). Briefly, this method involves partitioning soil into twin 0.2 g samples to which known volumes of ^{18}O enriched water or unlabeled water equivalent are added. These samples are incubated for 24 h and the CO_2 respired by soil microbes in that period is measured. On the same day as the onset of incubation, a

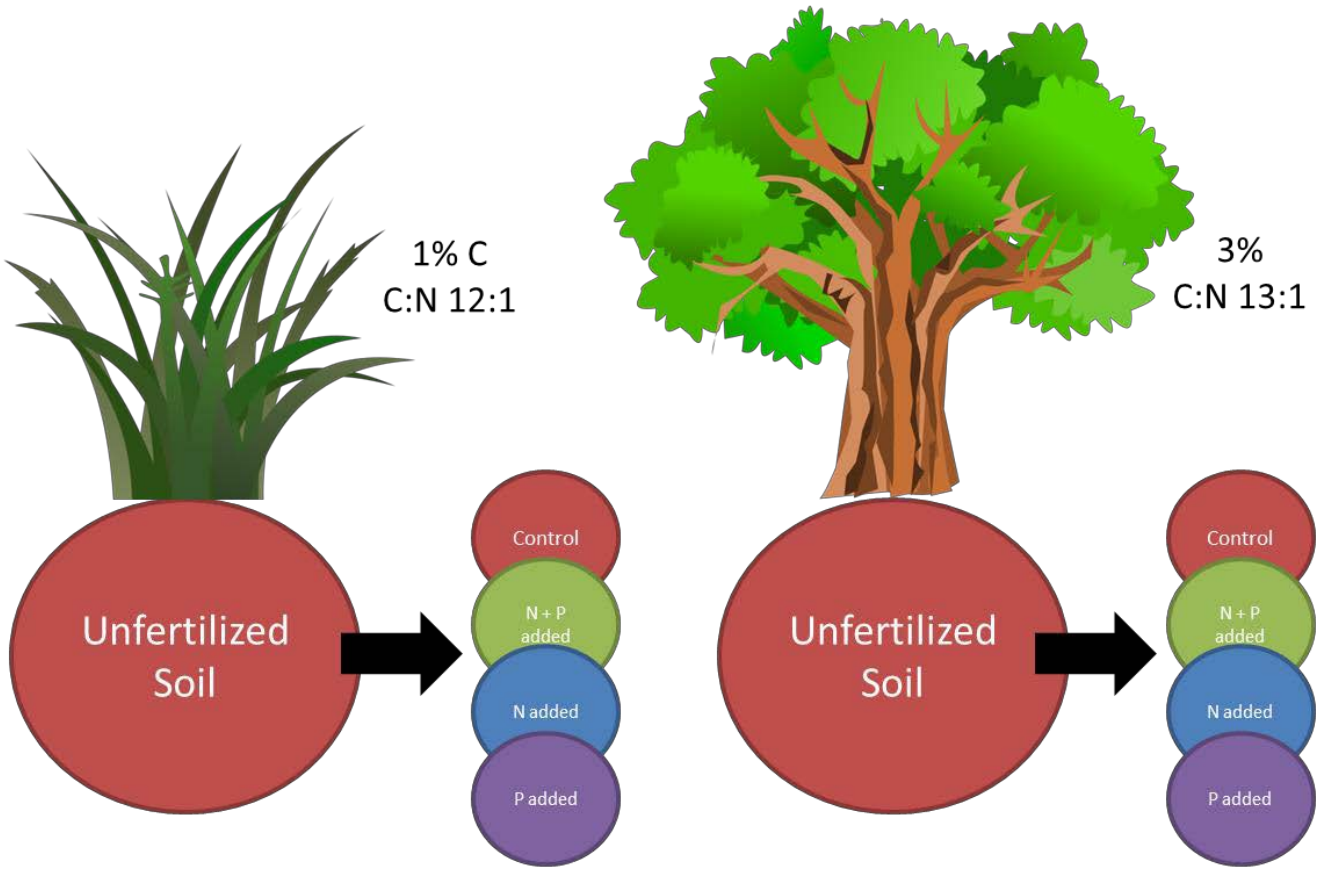

Figure 1. Conceptual schematic for proximate nutrient addition of soil from open grassland areas (relatively low SOM) and under tree canopies (relatively high SOM) of an oak-savanna.

chloroform fumigation extraction (CFE) is performed to determine the microbial biomass C g^{-1} soil for each sample (Brookes et al., 1985). At the end of the incubation the twin sets of labeled and unlabeled soil are flash frozen and subsequently DNA extracted. By measuring the excess ^{18}O content of microbial DNA, and assuming that this value comes from DNA newly-built during the 24 h incubation, one can calculate the microbial biomass C that would have developed alongside that DNA using the ratio between microbial biomass (from CFE procedure, in C g^{-1}soil) and DNA (in ng g^{-1} soil). This calculated value for biomass C is then combined with the known CO_2 respired for the sample and CUE is calculated as follows, where $C_{biomass}$ equals the calculated biomass built over 24 h and $C_{respired}$ equals C from microbial respiration over 24 h (see below):

$$CUE = C_{biomass} / (C_{biomass} + C_{respired})$$

Samples were labeled using either 95% or 100% ^{18}O water, which resulted in a final enrichment of soil water ranging from 27-38% ^{18}O. Headspace samples were collected at the beginning and end of the 24 h incubation and run on a Delta V™ Advantage IRMS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to determine $C_{respired}$.

DNA was extracted using a kit (FastDNA™ Soil DNA extraction kit, MP Biomedical, Santa Ana, California, USA) and the concentration was determined using a fluorescence assay (Quant-iT™ PicoGreen™ dsDNA assay kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The samples were then run on a Delta V™ Advantage IRMS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to determine their ^{18}O content.

CFE was performed using 30 mL of 1 M KCl and 4 g soil (fresh weight). Pre-incubated samples were fumigated with chloroform in the dark for 24 h. Extracts from fumigated and unfumigated subsamples were run for total organic C and total organic N on a TOC-VCPH/TNM-1 (Schimadzu Corp., Kyoto, Japan), hereafter referred to as EOC (extractable organic C) and EON (extractable organic N). Microbial biomass

Table 1. Salts used to establish proximate nutrient addition treatments. The masses listed here were applied to soil (1.1 & 0.92 kg dry soil equivalent in open grassland and under canopy habitat soils, respectively), dissolved in volumes of distilled water calculated to bring soil to 60% of the water-holding capacity. Differences in soil mass are due to different bulk densities of the two soils. Salts were selected to maintain an equal potassium (K) fertilization effect in all treatments.

Nutrient Addition Treatment	N containing salts added	mmol of N added	P containing salt added	mmol of P added
+N+P	0.148 g NH_4NO_3	3.7	0.114 g KH_2PO_4	0.8
+N	0.085 g KNO_3, 0.115 g NH_4NO_3	3.7 = 0.8 + 2.9	-	
+P	-		0.114 g KH_2PO_4	0.8

C and N were calculated as the difference between fumigated and unfumigated samples. Extractable P and extractable microbial P were determined on finely ground dry soil (from the gravimetric water content of the CFE procedure) by extracting paired 0.5 g samples, one of which was slurried in 1 mL of chloroform for 1 hour prior, in 0.5 M $NaHCO_3$ for 16 h. This is a modification of the method of Hedley and Stewart (1982) in that we extracted soil in water before slurrying with chloroform, but we did not remove resin-available phosphate (PO_4^{-3}) in this step, as no resin strips were included in the water. Analysis of PO_4^{-3} concentrations in the 0.5 M $NaHCO_3$ supernatant was done on a Lachat QuickChem 8500 Series2 (Lachat, Hach, Loveland, CO, USA). Microbial P was calculated as the difference in total P as determined by ICP analysis between slurried and unslurried samples using a *k* factor of 0.35 (Cleveland and Liptzin, 2007).

Enzyme Activity

For all samples we measured the activity of β-glucosidase, NAG, phosphatase, and sulfatase using a multi-substrate modification of Marx et al. (2001). This is a fluorimetric assay using 4-methylumbelliferone (MUF), bonded to enzyme-specific substrates. A small (1 g, 60% WHC) subsample was dispersed with 50 mL of sterile water, 50 µl of this solution was pipetted into 6 cells of a black 96-cell tray. These 6 cells then received 50 µl of 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer and 50 µl of MUF-bonded substrate. Plates were incubated at 30°C in the dark for 210 min, with fluorescence measurements at 360 nm excitation and 460 nm emission taking place after 30, 60, 90, 150, and 210 min on a Microplate Fluorescence Reader FLx800 ® (Bio-Tek Instruments, Inc., Winooski, VT, USA). Peroxidase and phenoloxidase activity was also measured using a near-identical assay, but instead of MUF and MES, Tetramethylbenzidin (TEM) and a 50 mM sodium acetate buffer were used with 200 µl of soil water suspension in a clear 96-cell plate. Results are reported as on a microbial biomass basis, as nmol MUF mg^{-1} microbial C h^{-1} or nmol TEM μg^{-1} microbial C h^{-1}.

^{14}C Incubation

To determine the radiocarbon age of substrates catabolized by soil microbes, 80 or 100 g of soil (for under canopy and open grassland, respectively) were incubated in the dark at 20˚C after a four-day pre-incubation. Samples were placed in 1 L mason jars which were then flushed with pure N_2 to remove any background C. Headspace gas was sampled after 3, 5, 7, and 10 days of incubation as needed to determine whether sufficient CO_2 (≥ 1 mg C per jar) had accumulated in incubation jars for subsequent extraction of CO_2 for ^{14}C analysis. As soon as sufficient C was present in the headspace, gas samples were

graphitized and run on a 3MV Tandetron Accelerator (HVEE, Amersfoort, Netherlands) at the ^{14}C analytical facilities in Jena, Germany (Steinhof et al., 2017).

Microbial Community Analysis

One replicate each from the control, +N+P (proximate), and +N+P (historical) treatments from both habitats was selected for DNA extraction and subsequent 16s rRNA gene sequencing. Frozen (-20°C) samples were removed from the freezer, and a 0.2 g subsample was extracted using the DNeasy Power Soil Kit (Qiagen, Venlo, Netherlands). PCR was performed on triplicate 0.5 µl aliquots of extraction products with the following specifications: 4.5 µl PCR water, 7.5 µl HotStart polymerase, 0.5 µl basepair Master Mix, 1µl forward and reverse 16S primer (341F and 785R, respectively, all materials from Qiagen, Venlo, Netherlands), for 30 cycles (denaturation 45 s at 94°C, annealing 45 s at 55°C, elongation 1 min at 72°C), and a final elongation of 10 min. Gel electrophoresis was performed to check the quality of PCR products. Sufficient amplification had taken place in all samples for sequencing. The final band was cut out of the gel and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). PCR products were sent for Illumina MiSeq sequencing (2x 300 bp paired end, LGC, Berlin, Germany). Raw sequences were quality controlled using FastQC version 0.11.8 (Andrews, 2019), then joined, merged, and demultiplexed using PEAR version 0.9.8 (Stamatakis et al., 2013). Sequences were denoised and chimeras removed using dada2 in qiime2 version 2019.4 (Callahan et al., 2016; Bolyen et al., 2018). A phylogenetic tree was constructed using the fragment-insertion technique (Janssen et al., 2018) comparing to the Greengenes 13_8 99% classifier (McDonald et al., 2011). Alpha-diversity was compared using observed OTUs for absolute α-diversity as well as Faith's Phylogenetic diversity which takes into account relatedness of OTUs and Pielou's evenness to look at the spread of OTUs (Pielou, 1966; Faith, 1992). Beta-diversity was assessed using weighted UniFrac, Sokal Michener, and Yule methods which were also used to generate data for principle coordinate analysis (Chang et al., 2011; Lozupone et al., 2011; Chen et al., 2012; Janssen et al., 2018) after samples were rarefied (subsampled without replacement) to 55,000 sequences per sample.

Statistical Analysis

To compare the effect of nutrient addition treatment (control, +N+P, +N, or +P) and habitat on response variables, data were analyzed using two-way ANOVA with the *car* package in R (Fox and Weisberg, 2019; R Core Team, 2019). We modeled these two factors as fixed-effects and allowed for interactions. Prior to analysis, data were transformed as needed to meet the assumptions of the ANOVA

method. This was done using the function transform Tukey from *rcompanion* (Mangiafico, 2019). If effects were significant ($p < 0.05$) differences between groups were tested using Tukey's HSD via the *emmeans* function from the same package (Lenth, 2019). One obvious outlier was removed from the +N treatment of the open grassland habitat (values 4x shifted from the other replicates). Differences in time since nutrient addition (control, proximate +N+P, and historical +N+P) were tested using a near-identical statistical set-up to that above with the obvious substitution of time for nutrient addition treatments.

Results

Soil Properties

Our proximate nutrient additions produced the expected shifts in N and P availability as evidenced by changes in extractable N:P ratios. Extractable N:P was higher under tree canopies (36 ± 3 vs 22 ± 3 in control samples, values are means ± se unless otherwise indicated). The N:P ratio of nutrient addition treatments ranked as follows: +N >> +N+P > Control > +P (Table 2). There was nearly twice as much microbial biomass C, N, and P in samples from under canopies, even when adjusted for soil C content (Table 2 & 3). Microbes in under canopies also tended towards a higher C:N ratio than microbes in open grassland soil. The two proximate N addition treatments (+N and +N+P) had higher than average microbial C:N in under canopy soil, although the effect was not significant

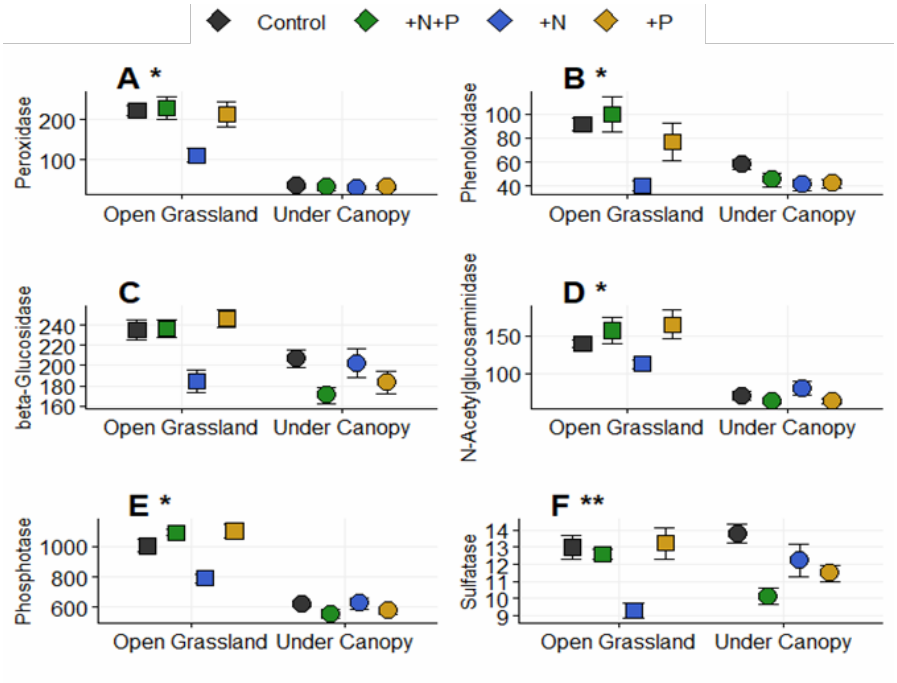

Figure 2. Potential enzyme activities for soil from open grassland (squares) and under tree canopies (circles) of the same dehesa with three different nutrient addition treatments. Values are means ± standard error (n = 4) of enzymatic reactions adjusted by mg microbial biomass C (nmol TEM μg^{-1} microbial C h^{-1} for A & B, nmol MUF mg^{-1} microbial C h^{-1} for C - F). * Denotes a significant difference between habitats. ** Indicates a significant interaction between habitat and nutrient addition treatments. Letters are Tukey groupings within a single habitat. Please note the scale of the y axis varies by plot.

(Table 2). One sample from the under canopy habitat in the control treatment had very high microbial biomass compared to the other replicates, but its inclusion did not affect the model output. Historically and proximately fertilized soil both had higher EOC compared to the control in open grassland soil (Table 3), while under canopies only the historical fertilization had higher EOC (Table 3).

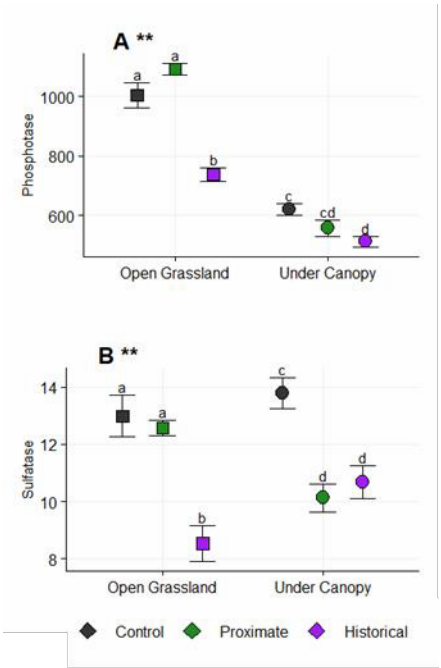

Figure 3. Potential enzyme activities for two soils from open grassland (squares) or under tree canopies (circles) from the same dehesa with proximate or historical addition of +N+P. Values are means ± standard error (n = 4) of enzymatic reactions adjusted by mg microbial biomass C (nmol TEM μg^{-1} microbial C h^{-1} for A, nmol MUF mg^{-1} microbial C h^{-1} for B).. ** Indicates a significant interaction between habitat and time since nutrient addition. Because of heteroscedasticity in the phosphatase potential data, under canopy and open grassland soil were analyzed separately for Tukey groupings. Letters are Tukey groupings within a single soil habitat. Please note the scale of the y axis varies by plot.

Enzyme potentials

After adjusting for microbial biomass C, potential enzyme activity was nearly always higher in open grassland soil, despite lower microbial biomass (Fig. 2A-F). All enzyme activity potentials were significantly affected by habitat, except β-glucosidase where the effect was only near significance (Fig. 2C, p = 0.07). Phenoloxidase, β-glucosidase, NAG, and phosphatase potential differed amongst nutrient addition treatments in open grassland soil, but not under canopies, with the +N treatment showing consistently reduced activity.

Time since fertilization did not affect the potential activity of peroxidase, phenoloxidase, β-glucosidase, or NAG. There was a significant interaction between time and habitat in sulfatase activity, with reduced sulfatase activity with historical fertilization in open grassland soil (control and proximate are similar, Figure 3B), but reduced activity with either nutrient addition in under canopy soil (control activity potential higher than fertilized). Because of heteroscedasticity in the phosphatase-potential data when both soil SOM and time effects were tested, under canopy and open grassland soil were analyzed separately. For open grassland soil, historical fertilization reduced phosphatase activity (similar to sulfatase, Fig. 3A). For under canopy soil, historical fertilization significantly reduced phosphatase activity, but proximate fertilization phosphatase activity was intermediate between this and the control and did not differ significantly from either (Fig. 3A).

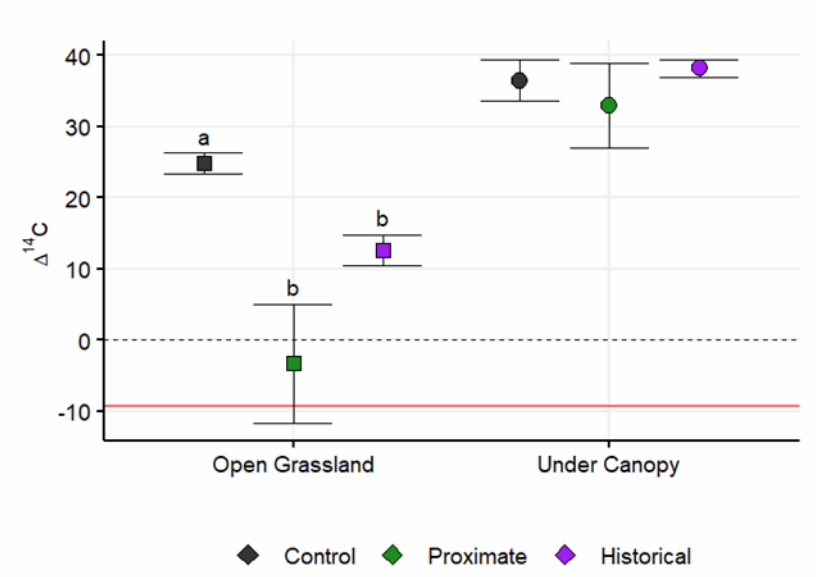

Figure 4. $\Delta^{14}C$ signature of respired CO_2 for two soils from open grassland or under tree canopies from the same dehesa with proximate or historical addition of +N+P. Red line represents the atmospheric $\Delta^{14}C$ value from the field site one year prior. Values are means ± standard error (n = 4). ** Indicates a significant interaction between habitat and time since nutrient addition. Letters are Tukey groupings within a single habitat.

$\Delta^{14}C$ of respired CO_2

Delta^{14}C of microbial respiration was significantly affected by habitat and proximate nutrient addition treatments, with no significant interaction. Under canopy soil had on average higher Δ values (35.3 ± 3.4‰) than open grassland soil (15.5 ± 3.6‰). Looking across habitats, the +N+P treatment (15.9 ± 8.5‰) had statistically lower Δ values than soil with +P (33.8 ± 4.0‰), but neither treatment differed from the control (30.7 ± 2.7‰) or +N soil (21.3 ± 5.8‰).

The $\Delta^{14}C$ signature of respired CO_2 was not significantly affected by time since fertilization in under canopy soils, although proximate fertilization did result in lower values (Figure 4). In the open grassland soil, both historical and proximate nutrient addition resulted in statistically lower delta values with the lowest values measured in the proximate nutrient addition (Fig. 4).

CUE and its components

All components of CUE and CUE itself differed between habitats (Figure 5A-D). Adjusted microbial respiration was higher in the open grassland soil, compared to under canopy soil (Fig. 5B). Within the open grassland samples, respiration was highest in the +P treatment, and lowest in +N, but these effects were not significant (Fig. 5B). Microbial growth rate and turnover was unaffected by P alone in under canopy soil, but changed significantly relative to the control in open grassland soil (lower growth rate, higher turnover, Fig. 5C&D). When N was added to under canopy soil, growth rate was reduced, but not significantly so (Fig. 5D). However, the effect of N addition on growth rate and turnover diverged with the presence or absence of P in open grassland soil, with addition of both (+N+P) resulting in the similar values as the control, and addition of N alone (+N) altering rates significantly compared to +N+P. As a result, CUE

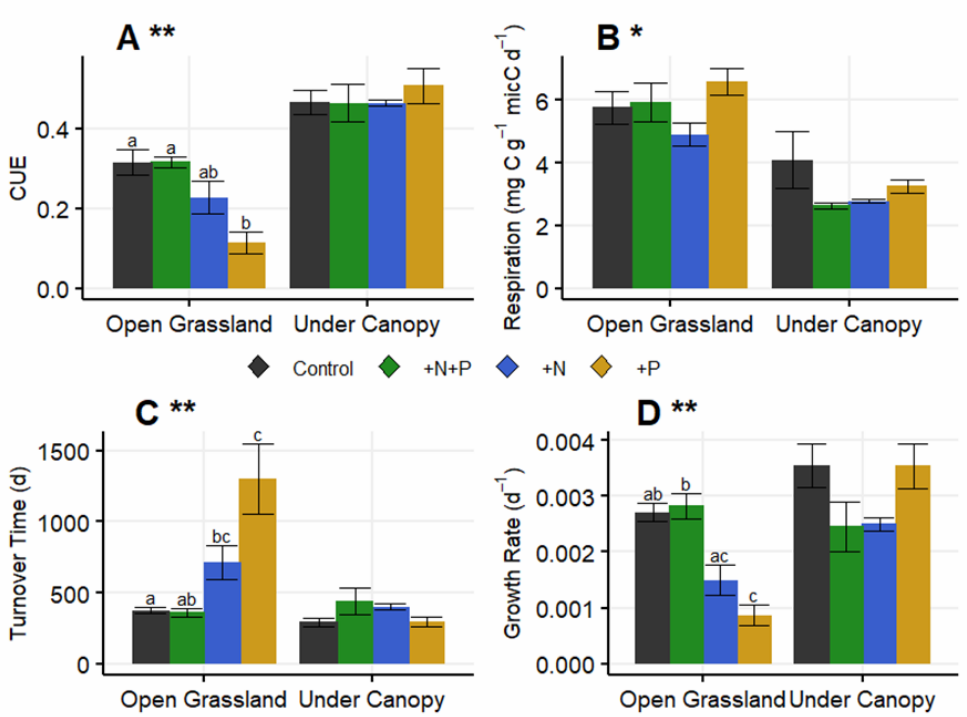

Figure 5. Effect of nutrient additions on A) CUE, B) respiration, C) turnover and D) mass specific growth rate of microbes from soil from open grassland and under tree canopy habitats within the same dehesa. Values are means ± standard error (n = 3-4). * Denotes a significant difference between habitats. ** Indicates a significant interaction between habitat and nutrient addition treatments. Letters are Tukey groupings within a single habitat. Please note the scale of the y axis varies by plot.

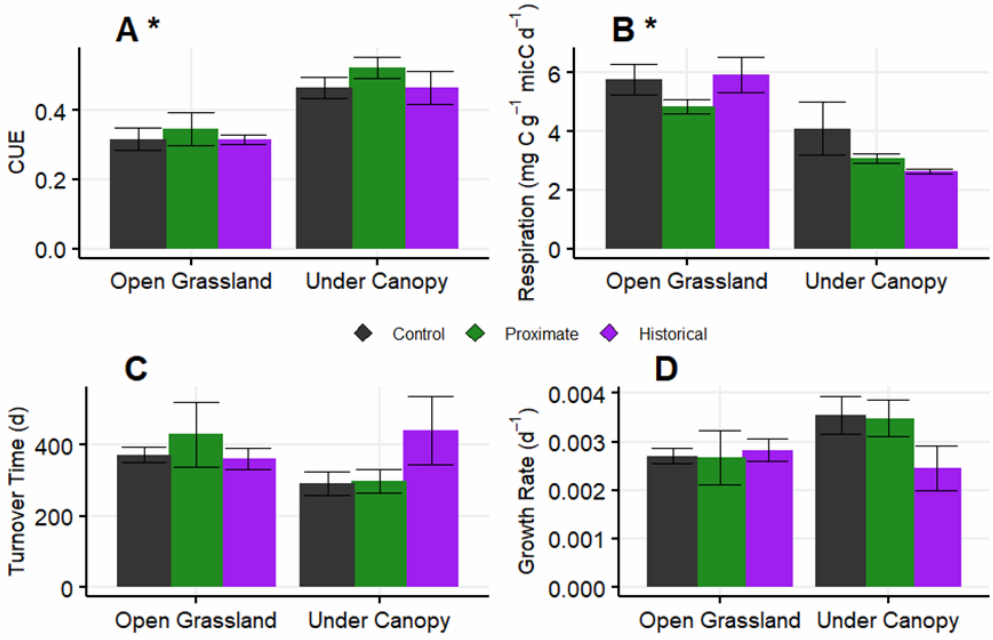

Figure 6. Effect of time since nutrient addition on A) CUE, B) respiration, C) turnover and D) mass specific growth rate of soil microbes from open grassland or under tree canopies within the same dehesa with +N+P addition proximately or historically. Values are means ± standard error (n = 3-4). * Denotes a significant difference between habitats. CUE & respiration were significantly affected by habitat. Please note the scale of the y axis varies.

in under canopy soil was unaffected by any nutrient addition, but was significantly reduced in the +P treatment, and notably lower (although not significantly so) in the +N treatment in open grasslands (Fig. 5A).

Time since fertilization had less of an effect on CUE and its components than the manipulation of soil stoichiometry (Figure 6A-D). Historical fertilization tended to reduce respiration in both soils but these effects were not significant (Fig. 6B). Turnover and growth rate of the microbial biomass did not differ significantly amongst groups, and were fairly variable (Fig. 6C & D). Historically fertilized soil tended to have the highest CUE, although this effect was not statistically significant.

Microbial Community

Neither the absolute α-diversity (observed OTU's) nor the phylogenetic diversity (Faith's) of the prokaryotic community from the two habitats differed significantly, but the evenness of their species composition did (Pielou's evenness, p = 0.049), with under canopy soil having greater evenness. Habitat also had a near significant effect on β-diversity (p = 0.1, similar values for Sokal Michener, Weighted Unifrac, and Yule methods) and this can be seen in the principle coordinate analysis plot (Figure 7). Fertilization shifted the microbial community, with the proximate +N+P treatment differing more from the control than the historical addition (Fig. 7), although due to low sample size these shifts are not statistically significant. The shift due to proximate-addition was greater in open grassland soil than under canopy, and was most readily seen in the Acidobacteria which dropped from 12% of sequences to just 5% while the Actinobacteria increased in these same samples from 23% to 32% of sequences.

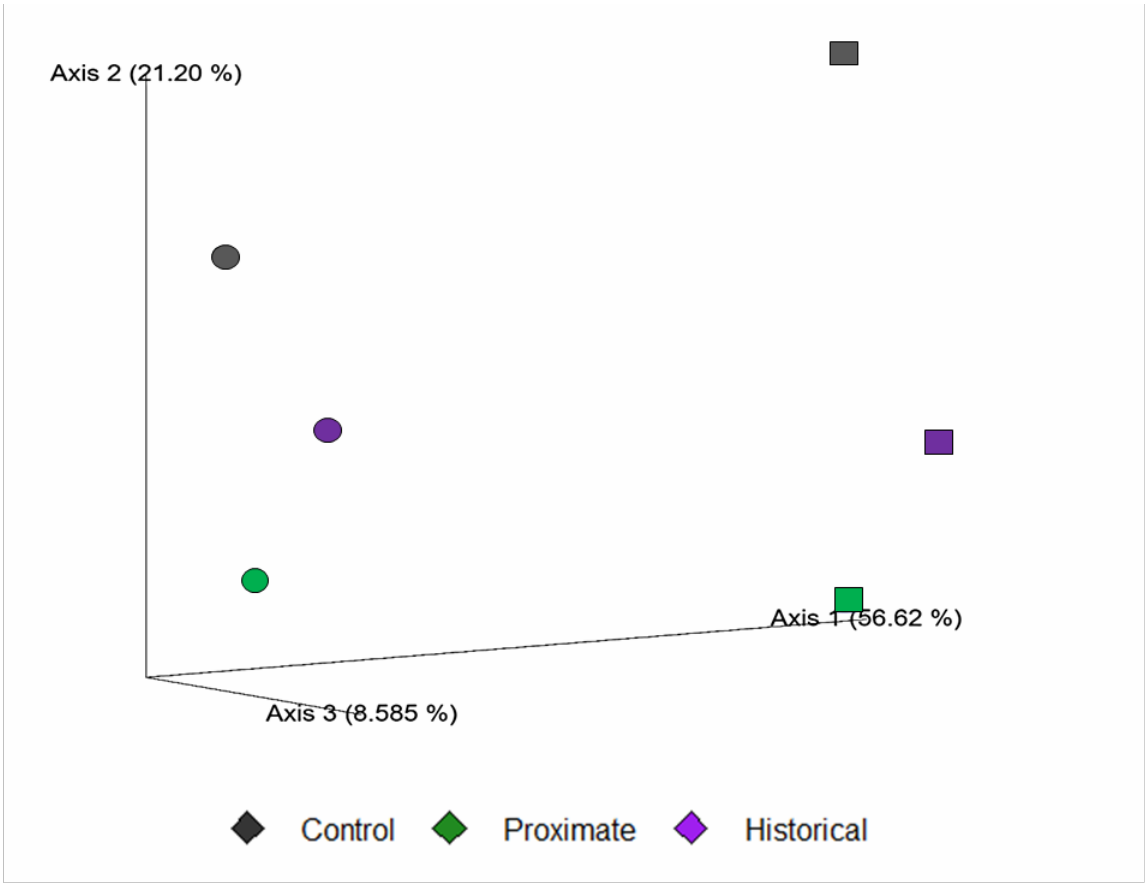

Figure 7. Principle coordinate analysis plot showing weighted-Unifrac β-diversity effect of time since nutrient addition on 16s RNA gene sequences in soil from open grassland (squares) or under tree canopies (circles) of the same dehesa with +N+P addition proximately or historically. Analysis is graphed using the EMPeror software package (Vázquez-Baeza et al., 2013).

Table 2. Extractable soil C, N, and P pools and their ratios for two surface soils from open areas and under tree canopies of a dehesa with three different nutrient addition treatments. Values are means ± standard error (n = 3-4). Capital letters are Tukey groupings for first order effects of treatment (across habitat), lower case letters are Tukey groupings for effects of treatment within one habitat (indicating a statistically significant interaction). Right-hand column indicates where habitat significantly affected pools and ratios. EOC & EON are extractable organic C or N values from 0.5 M K_2SO_4, P values from 0.5 M $NaHCO_3$ extracts respectively, see methods for more details.

	Open Grassland				Under Canopy				
	Control	+N+P	+N	+P	Control	+N+P	+N	+P	Habitat Effect
EOC mg g^{-1} C	34 ± 2.5 AB	44 ± 1.9 B	34 ± 2.0 A	43 ± 3.1 B	52 ± 2.6 AB	51 ± 2.5 B	44 ± 1.1 A	51 ± 1.5 B	***
EON µg g^{-1}	152 ± 9 a	675 ± 40 b	730 ± 31 b	170 ± 11 a	605 ± 50 c	1663 ± 70 d	1755 ± 31 d	538 ± 8 c	***
PO_4, P µg g^{-1}	8.1 ± 1.5 A	13 ± 0.4 B	5.0 ± 0.9 A	14 ± 3 B	17 ± 0.3 A	29 ± 0.9 B	16 ± 0.9 A	28 ± 0.4 B	***
extractable N:P†	21 ± 3 a	52 ± 3 b	156 ± 29 c	14 ± 2 a	36 ± 3 d	56 ± 4 d	109 ± 9 e	20 ± 1 f	**
Mic C mg g^{-1} C	166 ± 12	154 ± 11	174 ± 3	161 ± 5	255 ± 42	286 ± 4	293 ± 6	302 ± 5	$p = 0.06$
Mic C:N	6 ± 0.2	7 ± 1.7	6 ± 0.6	6 ± 0.1	7 ± 0.6	8 ± 0.4	8 ± 0.4	6 ± 0.1	-
Mic C:P‡	56 ± 26	40 ± 7	45 ± 4	44 ± 7	182 ± 88	163 ± 7	206 ± 62	281 ± 44	***

†P values in this ratio are from 0.5 M $NaHCO_3$ extractable PO_4 only

‡P values in this ratio are from 0.5 M $NaHCO_3$ extractable P only

Table 3. Extractable soil C, N, and P pools and their ratios for two surface soils from open areas and under tree canopies of a dehesa with either proximate or historical addition of +N+P. Values are means ± standard error (n = 3-4). Capital letters are Tukey groupings for first order effects of time since nutrient addition (across habitat), lower case letters are Tukey groupings for effects of time within one habitat (indicating a statistically significant interaction). Right-hand column indicates where habitat significantly affected pools and ratios. EOC & EON are extractable organic C or N values from from 0.5 M K_2SO_4, P values from 0.5 M $NaHCO_3$ extracts respectively, see methods for more details.

	Open Grassland			Under Canopy			
	Control	Proximate	Historical	Control	Proximate	Historical	Habitat Effect
EOC mg g^{-1} C	34 ± 2.5 A	44 ± 1.9 AB	44 ± 0.58 B	52 ± 2.6 A	51 ± 2.5 AB	60 ± 3.3 B	***
EON µg g^{-1}	152 ± 9 a	675 ± 40 b	230 ± 8 c	605 ± 50 d	1663 ± 70 e	585 ± 20 d	***
PO_4, P µg g^{-1}	8.1 ± 1.5 A	13 ± 0.4 B	19 ± 0.4 C	17 ± 0.3 A	29 ± 0.9 B	57 ± 5.7 C	***
extractable N:P†	21 ± 3 a	52 ± 3 b	12 ± 0.4 c	36 ± 3 d	56 ± 4 e	11 ± 1 f	***
Mic C mg g^{-1} C	166 ± 12 A	154 ± 11 A	259 ± 3 B	255 ± 42 A	286 ± 4 A	342 ± 4 B	**
Mic C:N	6 ± 0.2	7 ± 1.7	7 ± 0.1	7 ± 0.6	8 ± 0.4	6 ± 0.1	-
Mic C:P‡	56 ± 26 AB	40 ± 7 A	67 ± 5 B	182 ± 88 AB	163 ± 7 A	326 ± 105 B	***

†P values in this ratio are from 0.5 M $NaHCO_3$ extractable PO_4 only

‡P values in this ratio are from 0.5 M $NaHCO_3$ extractable P only

Discussion

4.1 Differences in soil habitats

Our results are in line with previous estimates of soil microbial CUE (Manzoni et al., 2012; Lee and Schmidt, 2014). Our lowest values are around 0.2, which is on the low end, and our highest values are around 0.6, which is just above the average for the field. We hypothesized that under canopy soil microbes would have higher CUE than those in open grassland. This was strongly supported by our results (Fig. 5A) with under canopy values about 66% greater than open grassland values in the control treatment. This supports the idea that CUE increases with the availability of C, because C content under tree canopies is also roughly 66% greater than in open grassland (Fig. 1). There is a good deal of evidence from the literature that soil microbes are C limited (Hobbie and Hobbie, 2013; Poeplau et al., 2016; Soong et al., 2018); however, it is possible that this difference in CUE is not due solely to greater SOM content. Under tree canopy soil also likely has a greater diversity of C substrates (Quideau et al., 2001; Vancampenhout et al., 2009), which could support higher CUE. It could also be that the community under tree canopies simply has greater CUE as an emergent property of its collective proteome. Enzyme activity per microbial biomass was lower under canopies, suggesting that reduced enzyme production might indeed result in higher CUE (*e.g.*, Manzoni et al., 2012). Likely it is a combination of all of these mechanisms and more specific data would need to be collected to parse the exact contributions.

4.2 Response of under canopy soil to proximate nutrient additions

We expected that CUE would increase with nutrient addition and that this increase would be associated with reduced enzyme activity. Focusing for the moment on the results from the under canopy soil, we did see modest reductions in enzyme activity (Fig. 2 and 3), but this was not associated with significant changes in CUE. This further supports the idea that microbes are dominantly C limited or alternatively, that production of enzymes (at least in the under canopy habitat) does not significantly take away from the energy that can be put in to building new biomass. There were slight (not statistically significant) reductions in microbial growth rate with N addition (both +N and +N+P) under canopies, but no change in CUE. Contrary to what one might expect from the treatment, these samples had higher C:N ratios of their microbial biomass (Table 2), so this reduction in growth rate may be associated with an increased abundance of the fungal community, which is generally slower-growing than the bacterial component (Rousk and Bååth, 2011) and tends towards higher C:N (Cleveland and Liptzin, 2007). There is conflicting evidence from the literature in regards to how fungi should respond to N addition in a semiarid

environment (Allison et al., 2007; Mueller et al., 2015) and because our microbial community analysis only targeted prokaryotes, additional study would be needed.

4.3 Effect of short-term balanced and imbalanced nutrient additions on open grassland soil

Contrary to our expectations, there were clear differential effects of +P, +N, and +N+P treatments in the open grassland habitat. We hypothesized that nutrient additions would result in a relief of nutrient limitation, however the increase in growth and CUE seen with +N+P in open grassland, whether historical or proximate, was minor and not statistically significant (Fig. 6A). Instead, CUE decreased markedly with imbalanced nutrient addition (Fig. 5A). While differential effects of N addition from N and P addition and even the addition of P alone are not uncommon (Olander and Vitousek, 2000; Liu et al., 2013; Poeplau et al., 2016; Mooshammer et al., 2017), mechanistic understanding of these differences remains tentative at best. One explanation proposed is that the stimulatory effect of P addition and the inhibitory effect of N addition cancel each other out (Olander and Vitousek, 2000), although this stimulatory effect of P addition is not seen in our data. Previous studies looking at N addition effects postulate that reductions in CUE correspond to reductions in oxidative enzyme activity (Carreiro et al., 2000; Cusack et al., 2010; Riggs and Hobbie, 2016), a concept supported here by reduced enzyme activity in the +N samples (Fig. 2A & B). If this reduction is due to inhibition, rather than decreased enzyme production, no change in CUE would be expected (Olander and Vitousek, 2000; Ramirez et al., 2012). However, there is no immediately apparent reason as to why this mechanism should be triggered only in the open grassland habitat of our study.

Given this surprising result, it is prudent to revisit the underlying assumptions of the ^{18}O method. The primary goal of our experiment was to explain the effect of nutrient addition on microbial CUE. Because identical nutrient additions were made to two distinct soil types, and only one of these showed an unanticipated response to the treatments, it seems unlikely that the nutrient additions led to violations of the method assumptions (*i.e.*, in that instance, we would have found the effect in both soil types). Despite this, two assumptions will be briefly considered. The first is that microbial community members contribute consistently to the emergent property that is microbial CUE over the period of measurement. In this instance the fungal to bacteria ratio of soil plays a major role (Pold et al, 2019), because fungi and bacteria have very different quantities of DNA per unit biomass and will respond to perturbations in potentially different ways (Demoling et al., 2008; Zhong et al., 2010). Additional samples from these two habitats collected in May of 2018 have shown that in untreated soil the open grassland actually has greater fungal:bacterial ratios than the under canopy habitat (Gogesch, 2019), although this might vary

seasonally. While this is more likely to affect the validity of comparing CUE values across habitats, it is possible that fungi within the open grassland habitat increased in abundance, which reduced our calculated CUE when P or N alone were added. Typically, decreases in fungal abundance post-fertilization are found in field studies (Allison et al., 2007; Demoling et al., 2008; Wang et al., 2018), although the effect is often not seen in semiarid sites (Mueller et al., 2015; McHugh et al., 2017). This explanation is relatively unsatisfying however, when one takes into account that the same mechanism somehow was not invoked when N and P were added together, nor in the under canopy habitat, and that it is unsupported by any changes in microbial biomass C:N. Therefore, we propose that differential responses of fungi and bacteria play a minor role in explaining decreased CUE in open grassland soil when single nutrients were added.

The second underlying assumption of the ^{18}O method to consider for our study is that intracellular water is in isotopic equilibrium with extracellular water (Blazewicz and Schwartz, 2011; Spohn et al., 2016a). Soils from under canopy and open grassland habitats have different SOM contents, and thus different water holding capacities. Samples were wetted to 60% of their individual water-holding capacity, implying that water dynamics at the soil-aggregate scale should have been similar (and non-limiting). However, if treatments affected how much intracellular-water was used to make new DNA, this DNA bias may have reduced the calculated CUE values (Li et al., 2016; Pold et al., 2019). Previous work has found that metabolic water contributes up to 40% of the PO_4-O in newly formed DNA and biomass for bacteria grown in pure culture (Li et al., 2016). Therefore, not only the degree of microbial activity but the types of metabolic reactions taking place within the cell would have a major impact on how accurate the intracellular-water assumption is. Although this seems like a promising line of research to enhance our understanding of microbial metabolism, it is beyond the scope of this project to determine. Consequently, we rely again on the evidence that chemically identical additions of N and P to under canopy soil, as well as the co-addition of these nutrients to open grassland soil, do not appear to have disrupted microbial metabolism sufficiently to interfere with this assumption. We therefore move forward under the premise that our results are a true reflection of reduced microbial growth.

Our strongest clue to untangling what happened to reduce open grassland microbial growth and CUE with imbalanced nutrient additions is in the $\Delta^{14}C$ signature of the respired CO_2. Addition of +N+P led to a significant shift in the C-substrate that microbes were using. It is known that addition of PO_4^{-3} releases SOM previously bound to mineral surfaces (Spohn and Schleuss, 2019), but we found no change in the $\Delta^{14}C$ signature of CO_2 in the +P treatment, implying that addition of both nutrients was required for microbes to utilize and release any additional SOM. A major difference between the measurement of $\Delta^{14}C$ signature of the respired CO_2 and that of microbial CUE is that the isotopic signature integrates C that is

respired over multiple days, whereas the CUE measurement integrates microbial activity over just 24 hours. All open grassland samples required 9 days of incubation, after a 4 day pre-incubation where the samples exchanged gas freely with the atmosphere, before sufficient C was respired for ^{14}C analysis. The CUE procedure was initiated after a 5 day pre-incubation, so these values are most representative of microbial growth at the beginning of the radiocarbon incubation. At that time, respiration rates were similar for all treatments, but growth was reduced in the single nutrient addition treatments. Microbial growth is at its foundation regulated by gene expression and this expression is controlled by complex feedbacks with the intra and extracellular environment (Chubukov et al., 2014). It seems the addition of both N and P allowed microbes to not only grow on different C substrates than those that received N or P alone, but also prevented the down-regulation of enzyme activity that we see in the N only treatment.

Reduced enzyme activity offers a clear explanation as to why the +N treatment had lower growth and CUE. But this is not seen when P alone was added. Phosphate is a pervasive biochemical modifier, as well as a key structural macronutrient which is present in cells in abundance (Jiménez et al., 2017). Polyphosphate (polyP) is a ubiquitous intracellular PO_4^{-3} storage compound that bacteria produce in response to stress (Rao et al., 2009; Albi and Serrano, 2016; Jiménez et al., 2017). This response is especially common in the instance of oxidative stress (Ault-Riché et al., 1998; Gray and Jakob, 2015). If we consider the open grassland soil to be a relatively stressful environment compared to the under canopy soil (supported by much greater peroxidase activity in open grassland samples), we might reasonably expect greater polyP accumulation in microbes there, especially in response to P addition. It seems plausible that polyP production would be triggered in the +P treatment and not the +N+P treatment due to the imbalance of nutrients. Furthermore, luxury uptake of P and subsequent formation of polyP is well-documented in algae and various bacterial species (Khoshmanesh et al., 2002; Eixler et al., 2006; Brown and Shilton, 2014). Unfortunately, we are not able to measure polyP in our samples, because methods for determining polyP concentrations are still in the early stages of development (Ault-Riché et al., 1998). We propose this hypothesis mainly because polyP formation is an energy consuming process, but it does not change the absolute amount of biomass P. Its formation could explain why the open grassland +P treatment had a trend of higher respiration and normal enzyme activity, but low CUE and similar microbial C:P to the +N+P treatment. ATP needed for anabolism might have been temporarily deflected into producing polyP. In general, the role of storage compounds in soil microbes and their influence on biogeochemical cycles has been neglected. Our result presents an exciting future line of research which can incorporate the two.

Table 4. Surface soil (0-5 cm) pH from four nutrient addition treatments and two distinct habitats of a dehesa (n = 4-6) as determined using a 1:1 slurry with 0.01 M $CaCl_2$. Values vary significantly by habitat but not by treatment.

Plot	Habitat	mean	±	sd
Control	Open Grassland	6.5	±	0.1
	Under Canopy	6.1	±	0.4
N added	Open Grassland	5.7	±	0.1
	Under Canopy	5.8	±	0.2
N+P added	Open Grassland	5.8	±	0.1
	Under Canopy	5.8	±	0.2
P added	Open Grassland	6.2	±	0.1
	Under Canopy	6.0	±	0.2
N+P added (historical)	Open Grassland	5.8	±	0.2
	Under Canopy	5.5	±	0.4

4.4 Differences in short vs long-term +N+P

Studies using soil from long-term fertilization experiments (> 10 years) generally show an increase in CUE with fertilization (Spohn et al., 2016b; Poeplau et al., 2019). Using the same ^{18}O method for measuring CUE, increases 30% - 40% above the control are reported when N and P were present in fertilizer. Interestingly, although we did find slightly (but not statistically significant) higher CUE in historically fertilized plots, the effect was nowhere near this magnitude (Fig. 6A). Our fertilized plot has only a moderate to weak history of fertilization compared to other studies, having been fertilized for 2 consecutive years with the last fertilization event occurring 2 years before soil was collected for this experiment. This is likely the reason for our modest increase in CUE. However, key to interpreting this result is the fact that proximate +N+P addition had no effect on CUE (Fig. 6A), and we know that our fertilized plot (historical +N+P) has had greater plant productivity than the control plot since the onset of fertilization treatments (Luo et al., 2018; Nair et al., 2019). This suggests that the true mechanism responsible for increased CUE is not changes in microbial processing of C, but plant responses to fertilization, increasing the amount of C available to microbes. Spohn et al. (2016b) also report the productivity of their plots, which scales well with the treatments showing increased CUE. We hypothesized that long-term +N+P treatment would show shifts in the C-substrates utilized by soil microbes, but we did not see any effect of historical fertilization on the in $\Delta^{14}C$ signature of the respired CO_2 (Fig. 4). This is potentially due to there being few changes in the plant community in response to fertilization, or these changes not being apparent in the soil C isotopic signature after only 3 years since the onset of fertilization treatments. An analysis of the plant community response to fertilization is forthcoming.

Another prediction of our study is that with fertilization, microbial communities shift over time (Ramirez et al., 2012). However, our analysis of the prokaryotic community does not support this as a mechanism. Proximate nutrient addition shifted the β-diversity of the community on the third axis (Fig. 7) relative to the control and historical fertilization, suggesting that the proximate nutrient addition had an effect on the prokaryotic community nearly equal in magnitude to that of historical fertilization. It also means that our final hypothesis regarding long-term community shifts in response to fertilization is only partially supported: communities are different in the long-term, but the change is very rapid and recovery

is very slow. However, given the low sample size used for community analysis, definitive statements should not be made.

4.5 Conclusion and future directions

We found strong evidence that microbial CUE at our sites is not nutrient limited, rather that CUE is limited by the amount (and likely quality and diversity) of C available to soil microbes. This finding sheds important light on understanding long-term fertilization effects on CUE, because changes in plant productivity likely play a greater role than increased availability of nutrients in determining this ecosystem property. Our unexpected finding of differential effects of imbalanced versus balanced nutrient addition is an exciting reminder that many aspects of soil microbial physiology remain unknown to us. Greater mechanistic understanding is urgently needed with regards to N and P uptake and how they interact to modify microbial activity and even gene expression. We encourage any future studies looking at N and P fertilization effects to consider the influence of time and to measure microbial polyP content.

Acknowledgements

The data reported here were mostly collected during a research stay with the University of Vienna Terrestrial Ecosystem Research group financially supported by the International Max Planck Research School. A great deal of thanks goes to Eva Simon for her expertise with the ^{18}O CUE method. The authors also thank Jeff Beem-Miller, Somak Chowdhury, Fabian Gans, Falko Gutmann, Alison Hoyt, Jessica Heublein, Theresa Kloetzing, Shane Stoner, and the entire Jena ^{14}C facility for their assistance with enzyme activity, ^{14}C, and 16S taxonomic data collection. KM developed the original concept of the experiment, generated the majority of the data, and wrote the manuscript. All authors contributed to the experimental design and data interpretation.

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Chapter 5

5.1 General Discussion

The purpose of this thesis was to increase our understanding of soil C cycling in tree-grass ecosystems, specifically in regards to how it is influenced by N and P availability and shifts in N:P stoichiometry. In light of continued anthropogenic N enrichment of ecosystems (Galloway et al. 1994, Gomez-Casanovas et al. 2016), it is likely that some ecosystems are shifting from N limitation to P limitation (Peñuelas et al. 2012), with essentially unknown consequences for their soil processes. Dehesas are an ideal ecosystem to study the significance of this shift because they belong to the subset of semi-arid ecosystems which contribute disproportionately to interannual variability in the global C budget (Ahlström et al. 2015). A defining feature of dehesas, and an additional motivation for studying C cycling in this particular ecosystem, is their mixed vegetative cover of scattered trees dotting open grassland (Joffre et al. 1999). This presents the opportunity to compare responses to N and P addition of soils with different quantities of SOC, stemming from their different habitats of origin (Gallardo 2003). My work focused on specific research gaps on the response of soil respiration to N and P availability, how the addition of P influences the ecosystem allocation of N, and how N and P influence the activity of soil microbes in addition to how all of these processes are influenced by habitat.

In all three of my experiments, the difference between dehesa habitat types was stark, with differences in the magnitude of their response to fertilization when N:P stoichiometry was maintained (control and +N+P addition treatments) or contrasting responses when N:P stoichiometry was shifted (N or P alone added). Responses to nutrient addition were generally greater in the low SOC environment of the open grassland, demonstrating that this low-fertility environment is more reactive to changes in nutrient addition than the relatively high SOC environment under tree canopies. The importance of the N:P ratio of perturbation is evident even 3 years post-fertilization in the soil respiration rates (Data-Chapter 1), as well as in the short-term response of soil microbes (Data-Chapter 3). Combining all results, the strongest effects were seen when N alone was added, shifting the ecosystem towards P limitation. Given increased respiration (Data-Chapter 1), increased turnover of plant biomass N (Data-Chapter 2), and lower short-term microbial CUE (Data-Chapter 3), it is unsurprising that there is no evidence of a net gain in C storage in the N addition treatment of the open grassland. In contrast, the under canopy soil responded more like forest soil when N alone was added, with a trend of decreased soil respiration (based on the more spatially reliable manual-measurements, Data-Chapter 1), slower biomass N turnover (Data-Chapter 2), and unaltered microbial CUE (Data-Chapter 3). Together this could lead to modest increases

in SOC over time. However, it is important to bear in mind that the open grassland habitat comprises 80% of the ecosystem, so the overall response of the dehesa will most strongly reflect the response of the open areas.

5.1.1 Effect of N and P on soil respiration

In my first data-chapter, I examined the effect of N and P addition on soil respiration at the main MANIP experimental site. The general trend in ecosystems is for soil respiration to decrease with N addition, with this decrease being attributed to a reduction in belowground C allocation by plants and/or shifts in the microbial community (Janssens et al. 2010). The effect of N and P addition, or the addition of P alone, is less well-studied, with results in all possible directions (Torn et al. 2005, He and Dijkstra 2015, Huang et al. 2018). Given the strong effect that N addition had on most response variables compared to the P-containing treatments in my studies, it may be that this paucity of studies on P effects springs from positive result bias in the literature (Fanelli et al. 2017). Regardless, much additional work remains to be done in order to parse differences in biological responses to N and P addition compared to their addition as single nutrients. In their 2010 review, Janssens et al. found that 25% of the forests studied exhibited increased soil respiration in response to N addition, and they attributed this to those sites being young or having low nutrient soil. In nutrient-poor soils, like the open grassland habitat of the dehesa, increases in ecosystem productivity in response to fertilization can overwhelm all other drivers of soil respiration, resulting in an increase in soil respiration in response to fertilization. I found the open grassland, with relatively low soil fertility, had increased intact-soil respiration in response to N addition. Interestingly, we know that microbes responded by decreasing their respiration with this same treatment in the short-term (Data-Chapter 3). However, it is likely that this result is driven by increased root biomass in response to N addition, which is known to occur at the site (Nair et al. 2019). There was also increased soil respiration in the open grassland in the P containing fertilizations during the spring of 2017, but these results were not significant. While it is known that the +N+P treatment plot also had increased pasture productivity, the root response differed, with roots increasing in density more than with N alone (Nair et al. 2019). This may have contributed to slightly, but not significantly higher soil respiration in the P containing treatments.

5.1.2 Fate of added N

Given the strong effect of N alone on soil respiration, it is surprising that in my second data-chapter I found no strong effect of increased P availability on the fate of N additions. For this experiment

I tracked ecosystem N allocation into plant and soil pools and found that added N was distributed similarly in the two nutrient addition treatments (N only vs +N+P). My expectation was that addition of P would increase the retention of added N in living components of the ecosystem, that the open grassland habitat would retain more of the added N than under canopy, and that added N would shift over time from soil microbes to plants. The dominant finding of this chapter was that regardless of time since application, P availability, or habitat, soil retained most of the added N. However, the results from data-chapter one and data-chapter two are not as incongruous as they may initially seem. I believe that the primary reason for the response to N addition of soil respiration in data-chapter one is increased plant biomass due to fertilization. Because the plots for my second data-chapter were established in 2017, an exceptionally dry year, and two years after the main MANIP experiment was initiated, it is possible the response of plants to fertilization in the tracer experiment was constrained by the drought. We know that 2017 had lower productivity than 2016 for the main MANIP experimental plots (Luo et al. 2018). If soil respiration and ecosystem N allocation are both dominantly driven by plant biomass and ecosystem productivity, the dry year would have had substantial repercussions for the tracer experiment. Additional work across multiple years should be conducted to confirm or refute an effect of P availability on N allocation in dehesas.

5.1.3 Isolated microbial response

In my final data-chapter I examine the isolated response of dehesa soil microbes to changes in N:P stoichiometry via their CUE, enzyme activity, and $\Delta^{14}C$ signatures of their respiration. Microbial CUE is a critical control point in soil C cycling because it determines not only how much C is respired by soil microbes, but how much C will be retained within microbial biomass. This microbial biomass C has the potential to persist within the soil C pool for long periods (Liang et al. 2017). There is support in the literature that microbial CUE is nutrient-limited and that increased CUE would be accompanied by decreased enzyme activity (Manzoni et al. 2012), being the net result of an internal trade-off between resources put into the production of nutrient acquiring enzymes (which need structural C as well as high energy bonds to build), and putting the same resources directly into building new biomass. I found no evidence of nutrient limitation in dehesa microbes though, regardless of habitat. Instead, because CUE was higher in soil from under tree canopies, which is richer in SOC, I conclude that availability of C is the primary factor influencing microbial CUE in this ecosystem. As with my previous chapters, this indicates that plant productivity is the primary driving factor controlling soil C cycling, and sheds critical light on previous studies attributing changes in microbial CUE to increased nutrient availability in long-term fertilization experiments (*e.g.*, Spohn et al. 2016).

Future work on this question should focus on the time scale at which nutrient addition influences plant productivity and that at which microbial CUE responds. Given our unanticipated results of differential effects of imbalanced versus balanced N and P addition, it would be useful to repeat the experiment across a wider soil fertility gradient. Mechanistic understanding could be enhanced by further reducing the complexity of the study system and looking at transcriptomics, for example, in a laboratory culture experiment. The fields of soil microbiology and biogeochemistry are in great need of easier methods for identifying and quantifying storage compounds, because changes in microbial biomass are often not specific enough to address the mechanisms needed to parameterize biogeochemical models.

5.2 Outlook

In combination, my thesis results demonstrate that addition of N or P as single nutrients can have substantially different results than their addition together. Greater mechanistic understanding is urgently needed with regards to N and P uptake and how they interact to modify plant and microbial activity. Such understanding is most readily obtained from model systems, such as *Arabidopsis* and *Escherichia*, where biochemical and gene-regulation pathways are already well-quantified. I encourage any future studies looking at N and P fertilization effects to consider the influence of time-since addition as a major factor to vary and to design experiments which can test specific mechanisms.

5.2.1 Implications for C storage with changes in N:P stoichiometry

A main finding of my thesis work is that in the dehesa open grassland, soil C cycling processes change in a way that could make soil C more vulnerable to loss when soil stoichiometry shifts towards higher N:P. In fact, while not statistically significant, a drop in soil C:N ratio mostly caused by loss of soil C is visible in the N addition treatment of the MANIP experiment over time (see Figure 4, data-chapter 1). Because most dehesas are located in rural areas with low N deposition rates (García-Gómez et al. 2014), this potential loss may not be realized. This is of course dependent on future N deposition rates remaining similar to those currently experienced. Given the importance of this ecosystem type to variability in annual global C uptake, it would be worthwhile to continue to monitor N deposition in this region. Any losses in soil C, and the mechanisms driving them, must be more carefully quantified before they can be incorporated into global C models. This could be in the form of similar studies to those presented here in other dehesas throughout the Iberian Peninsula, but also in the form of soil C monitoring. A likely mechanism for C loss is P-mining by plant roots, since I found no evidence of P limitation for the microbial biomass. An alternative interpretation of my overall results is that soil C cycling is merely accelerated in

soil with increased N:P, in which case no C loss would be expected. A multi-year tracer experiment using ^{13}C would be able to distinguish between these two mechanisms.

5.2.2 Implications for dehesa management

N deposition is not the only way by which soil N:P stoichiometry can increase. Dehesas are actively managed ecosystems, and some are even routinely fertilized (Olea and San Miguel-Ayanz 2006). Given the results of my thesis, land managers should be encouraged to ensure that any fertilization is done using a balanced approach, and never with the addition of N alone. This may be a challenge because fertilizer is of course expensive, but also because increases in pasture productivity were similar whether N or N and P are added (El-Madany et al. 2018). However, if loss in soil C is realized when N alone is added, the magnitude of increased productivity will decrease with time in these fields, as soil fertility is decreased by loss of SOM. Another key message is that trees play a critical role in stabilizing the ecosystems response to environmental drivers, and increased tree cover would lead to increased ecosystem stability in the face of climate change.

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Summary

There is a critical need to increase our knowledge of how the availability of nutrients, namely nitrogen (N) and phosphorus (P), affects carbon (C) cycling (Zaehle et al. 2010, Peñuelas et al. 2012, Stiles et al. 2017). This need arises from increased availability of N and P due to anthropogenic activities, such as fossil fuel combustion and widespread fertilizer use, which have the potential to increase C stocks if these changes lead to increased plant productivity (Magnani et al. 2007, LeBauer and Treseder 2008). Whether or not increased productivity leads to increases in ecosystem C stocks is in large part dependent on whether processing of C by soil microbes is also increased by nutrient addition. Addition of N to soil generally reduces microbial biomass and enzyme activity (Treseder 2008, Ramirez et al. 2012), but the response of ecosystems to P addition is generally less studied (Vitousek et al. 2010) and it is unclear from our current understanding if there is a general microbial response to such additions (Liu et al. 2012, Huang et al. 2016). Often the results of experiments using co-addition of N and P differ from the addition of either as a single nutrient (Ramirez et al. 2012, Poeplau et al. 2016, Huang et al. 2018). Given expected shifts in N:P ratios in soils, due to differential rates of anthropogenic input (Peñuelas et al. 2012), increased understanding of how the stoichiometry of these nutrients influences soil C cycling is essential to understanding how ecosystem C stocks will respond. Likely, the response of soil C processes to changes in N:P availability depends on site fertility (Kang et al. 2016). To remove this barrier, experiments can be conducted in ecosystems that naturally contain soils with contrasting fertilities. In tree-grass ecosystems, such as oak-savannas, there are areas with higher concentrations of soil organic C (SOC) due to *in situ* decomposition of tree litter (Gallardo 2003, Moreno et al. 2007). The aim of this dissertation is to quantify how N and P availability affects soil C cycling in an oak-savanna using several approaches.

In the first data chapter, I investigated how soil respiration is affected by changes in N and P availability. Soil respiration is a combination of microbial respiration and respiration from plant roots, making it an excellent parameter for understanding the combined response of plants and soil microbes to environmental change. For this data chapter I measured soil respiration in sampling campaigns from March 2017 to May 2018 in a large-scale fertilization experiment including N, P, and N&P addition plots. I used data from hand-held measurements and automated chambers to model changes in the basal respiration and temperature sensitivity of soil respiration in response to changes in available N:P. We found that the overall behavior of soil respiration and its response to nutrient addition differed strongly between soils under tree canopies and those in open grassland areas. Open grassland showed significantly increased respiration with N addition whereas soil under tree canopies had a trend towards the opposite response. The response of soil respiration to N alone was much stronger than the response to N&P or P

alone, which means that this ecosystem is sensitive to an increase in the N:P ratio of available nutrients. My results confirm that SOC content is a predictor of how soil respiration responds to nutrient addition.

For my second data chapter, I investigated the role of P availability in the ecosystem allocation of added N fertilizer. This chapter reports on a field-based stable isotope tracer experiment that follows the uptake of added N by surface soil, plants, and soil microbes under tree canopies and in open grassland areas. In 2017 I created paired experimental plots with and without P addition and added ^{15}N to determine the fate of N over the period of one year. I found that open grassland with N alone had the largest label recovery seven months after N addition; this supported the idea that open grassland habitat is more N-limited than under canopy. However, soil was the largest sink for additional N, regardless of habitat, P addition, or time. My results suggest that abiotic fixation of N may play an important role in modifying the effects of N addition via anthropogenic activity in oak-savannas.

My third and final data chapter deals with changes in microbial activity after N and P addition, and how this changes over time. To isolate the microbial response, I performed a laboratory study where I measured the microbial carbon-use efficiency (CUE), as well as related variables such as enzyme activity and isotopic signature of respired C, on samples with short and long-term histories of fertilization. I found that nutrient addition had no effect on microbial CUE in the relatively high SOC habitat under tree canopies on both time scales. In contrast, the low SOC habitat of the open grassland had a lower CUE when single nutrients were added in the short-term, with significantly reduced CUE when P alone was added, but unchanged when N and P were combined. My results suggest that stoichiometric imbalances may reduce microbial CUE in the short term, but further studies are needed to determine how long such effects last. Overall, my results show that microbial activity in this oak-savanna is not nutrient-limited, but limited by the amount of C available for soil microbes.

A primary result of my work is that the soil in the open grassland component of oak-savannas is much more sensitive to changes in nutrient availability than that under tree canopies. In the grassland areas, soil C becomes more susceptible to losses when soil stoichiometry moves in the direction of higher N:P due to increased soil respiration. An alternative interpretation of my overall results is that the C cycling in soils with elevated N:P is merely accelerated, in which case no C loss would be expected. In combination, my thesis results show that the addition of N or P as individual nutrients can produce significantly different results compared to their addition together. A greater mechanistic understanding is urgently needed as to how N and P uptake is controlled in soil microbes and how these nutrients interact to alter plant and microbial activity.

Zusammenfassung

Es besteht die dringende Notwendigkeit, unser Wissen darüber zu erweitern, wie die Verfügbarkeit von Nährstoffen, nämlich Stickstoff (N) und Phosphor (P), den Kohlenstoff-(C)-Zyklus beeinflusst (Zaehle et al. 2010, Peñuelas et al. 2012, Stiles et al. 2017). Dieser Bedarf ergibt sich aus der erhöhten Verfügbarkeit von N und P aufgrund anthropogener Aktivitäten, wie der Verbrennung fossiler Brennstoffe und der weit verbreiteten Düngung, die das Potenzial haben, die C-Bestände zu erhöhen, wenn diese Veränderungen zu einer höheren Pflanzenproduktivität führen (Magnani et al. 2007, LeBauer und Treseder 2008). Ob eine erhöhte Produktivität zu einem Anstieg Kohlenstoffs eines Ökosystems führt oder nicht, hängt zu einem großen Teil davon ab, ob die Nutzung von C durch Bodenmikroben auch durch Nährstoffzugabe erhöht wird. Die Zugabe von N zum Boden reduziert im Allgemeinen die mikrobielle Biomasse- und Enzymaktivität (Treseder 2008, Ramirez et al. 2012), aber die Reaktion der Ökosysteme auf die P-Zugabe ist im Allgemeinen weniger untersucht (Vitousek et al. 2010) und es ist nach derzeitigen Verständnis unklar, ob es eine allgemeine mikrobielle Reaktion auf solche Zugaben gibt (Liu et al. 2012, Huang et al. 2016). Häufig unterscheiden sich die Ergebnisse von Experimenten mit gleichzeitiger Gabe von N und P von der Zugabe von entweder als Einzelnährstoff (Ramirez et al. 2012, Poeplau et al. 2016, Huang et al. 2018). Angesichts der erwarteten Verschiebungen des N:P-Verhältnisses in Böden, die auf unterschiedliche Raten des anthropogenen Eintrags zurückzuführen sind (Peñuelas et al. 2012), ist ein besseres Verständnis dafür, wie die Stöchiometrie dieser Nährstoffe den Kreislauf des Bodens beeinflusst, von wesentlicher Bedeutung, um zu verstehen, wie die C-Bestände des Ökosystems reagieren werden. Wahrscheinlich hängt die Reaktion von Boden-C-Prozessen auf Veränderungen der N:P-Verfügbarkeit von der Fruchtbarkeit des Standorts ab (Kang et al. 2016). Um diese Barriere zu beseitigen, können Experimente in Ökosystemen durchgeführt werden, die aufgrund ihrer Struktur Böden mit kontrastierenden Fruchtbarkeiten besitzen. In Baum-Gras-Ökosystemen, wie z.B. Eichensavannen, gibt es Gebiete mit höheren Konzentrationen an organischem C (SOC) im Boden durch den *in situ* Abbau von Baumstreu (Gallardo 2003, Moreno et al. 2007). Das Ziel dieser Dissertation ist es, mit verschiedenen Ansätzen zu quantifizieren, welche Auswirkungen die Verfügbarkeiten von N und P auf den Bodenkohlenstoffkreislauf in einer EichensSavanne haben.

Im zweiten Kapitel untersuchte ich, wie die Bodenatmung durch Veränderungen der N- und P-Verfügbarkeit beeinflusst wird. Die Bodenatmung ist die Summe aus mikrobieller Atmung und Atmung aus Pflanzenwurzeln, was sie zu einem ausgezeichneten Parameter für das Verständnis der kombinierten Reaktion von Pflanzen und Bodenmikroben auf Umweltveränderungen macht. Für dieses

Kapitel habe ich die Bodenatmung in Flächen eines großräumigen Düngeversuchs mit N-, P- und +N+P-Zugabe in Beprobungskampagnen von März 2017 bis Mai 2018 gemessen. Ich benutzte Daten aus manuellen Messungen und automatisierten Kammern, um Veränderungen der Basalatmung und der Temperatursensitivität der Bodenatmung als Reaktion auf Veränderungen des verfügbaren N:P zu modellieren. Wir fanden heraus, dass sich das Gesamtverhalten der Bodenatmung und ihre Reaktion auf die Nährstoffzufuhr zwischen Böden unter Baumkronen und solchen in offenen Grünlandgebieten stark unterschied. Offenes Grünland zeigte eine signifikant erhöhte Atmung bei N-Zugabe, während Boden unter Baumkronen einen Trend zum Gegenteil zeigte. Die Reaktion der Bodenatmung auf N allein war deutlich stärker als die Reaktion auf +N+P oder P allein, was bedeutet, dass dieses Ökosystem empfindlich auf eine Erhöhung des N:P-Verhältnisses der verfügbaren Nährstoffe reagiert. Meine Ergebnisse bestätigen, dass der SOC-Gehalt ein Indikator dafür ist, wie die Bodenatmung auf die Nährstoffzufuhr reagiert.

Für das dritte Kapitel untersuchte ich die Rolle der P-Verfügbarkeit bei der Ökosystemallokation von N-Dünger. Dieses Kapitel berichtet über ein Feldexperiment, das mithilfe von stabilen Isotopen die Aufnahme von zugesetztem N durch Oberboden, Pflanzen und Bodenmikroben in offenen Grünlandgebieten und unter Baumkronen nachverfolgt. Im Jahr 2017 habe ich Versuchsflächen mit und ohne P-Zusatz erstellt und 15N hinzugefügt, um den Verbleib von N über den Zeitraum von einem Jahr zu bestimmen. Ich fand heraus, dass offenes Grasland mit alleiniger N-Zugabe die größte Label-Rückgewinnung sieben Monate nach N-Zugabe hatte; dies unterstützte die Idee, dass Habitate in offenem Graslandstärker N-limitiert sind als solche unter Baumkronen. Allerdings war der Boden die größte Senke für zusätzliches N, unabhängig von Lebensraum, P-Verfügbarkeit oder Zeit. Meine Ergebnisse deuten darauf hin, dass die abiotische Fixierung von N eine wichtige Rolle bei der Modifikation der Effekte der N-Deposition in Eichen-Savannen spielen kann.

Mein viertes Kapitel beschäftigt sich mit Veränderungen der mikrobiellen Aktivität nach N- und P-Zugabe und wie sich diese im Laufe der Zeit ändert. Um die mikrobielle Reaktion zu isolieren, führte ich eine Laborstudie durch, in der ich die mikrobielle Kohlenstoff-Nutzungseffizienz (CUE) sowie damit eng verwandte Variablen wie Enzymaktivität und Isotopensignatur des aufgenommenen C an Proben mit kurz- und langfristig erfolgter Düngung gemessen habe. Ich fand heraus, dass die Nährstoffzugabe auf beiden Zeitskalen keinen Einfluss auf den mikrobielle CUE unter Baumkronen mit relativ hohen SOC-Werten hatte. Im Gegensatz dazu hatte der Lebensraum des offenen Grünlandes mit niedrigen SOC-Werten bei kurzfristiger Zugabe einzelner Nährstoffe einen niedrigeren CUE-Wert, mit deutlich

reduzierter CUE bei Zugabe von P allein, aber unveränderter CUE bei Kombination von N und P. Meine Ergebnisse deuten darauf hin, dass stöchiometrische Ungleichgewichte kurzfristig die mikrobielle CUE reduzieren können, aber weitere Studien sind notwendig, um festzustellen, wie lange solche Effekte anhalten. Insgesamt zeigen meine Ergebnisse, dass die mikrobielle Aktivität in dieser Eichen-Savanne nicht nährstoffbegrenzt ist, sondern durch die für Bodenmikroben verfügbare Menge an C begrenzt ist.

Ein primäres Ergebnis meiner Arbeit ist, dass der Boden im offenen Grünlandteil von Eichen-Savannen viel empfindlicher auf Veränderungen der Nährstoffverfügbarkeit reagiert als unter Baumkronen. In den Grünlandflächen wird der Boden-C anfälliger für Verluste, wenn sich die Bodenstöchiometrie aufgrund erhöhter Bodenatmung in Richtung höherer N:P-Werte bewegt. Jede Veränderung im Boden-C und den dafür verantwortlichen Mechanismen muss genauer quantifiziert werden, bevor er in globale C-Modelle aufgenommen werden kann. Eine alternative Interpretation meiner Gesamtergebnisse ist, dass der C-Kreislauf in Böden mit erhöhtem N:P lediglich beschleunigt wird, so dass in diesem Fall kein C-Verlust zu erwarten ist. In Kombination zeigen meine Ergebnisse, dass die Zugabe von N oder P als einzelne Nährstoffe zu signifikant unterschiedlichen Ergebnissen führen kann, verglichen mit ihrer gemeinsamen Zugabe. Ein größeres mechanistisches Verständnis ist dringend erforderlich, wie die N- und P-Aufnahme durch Bodenmikroben gesteuert wird und wie diese Nährstoffe interagieren, um die Aktivität von Pflanzen und Mikroben zu verändern.

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Objective: I am passionate about soil microbiology, biogeochemistry, and ecology. I love teaching and outreach, whether in the laboratory, the lecture hall, or the public sphere.

Education

PhD Ecology, Max Planck Institute for Biogeochemistry, Jena, Germany

in aff. with Friedrich-Schiller University, Jena anticipated defense March 2020

Dissertation Title:
Influence of nutrient availability on soil respiration and microbial activity in a tree-grass ecosystem

MS Ecology, Utah State University, Logan, UT, USA 2014

BS Biology, Trinity University, San Antonio, TX, USA 2009

Work Experience

Doctoral Researcher, Dept. of Biogeochemical Integration, MPI-BGC
January 2016 – present

- Support existing research lines and simultaneously develop novel projects to advance understanding of soil carbon cycling and how it is influenced by nitrogen (N) and phosphorus (P) fertilization in a semi-arid oak savanna, as part of the MANIP experiment framework (project website). Independently developed topics include a ^{15}N tracer experiment tracking fate of added fertilizer in the field, field campaigns examining N:P control on soil respiration using multiple approaches, and applying a cutting-edge ^{18}O -DNA labeling method for measuring microbial carbon use efficiency to assess how it varies with N and P availability.
- Supervise bachelor's and master's level assistants during data collection and sample processing.
- Mentored a bachelor's student thesis project titled: *Evaluation of microbial communities in a dehesa using PLFA and NLFA*, with plans to prepare this thesis for publication with the student.

Acting Director of Undergraduate Studies, Dept. of Biology, Utah State University
June – Dec. 2015

- Advised incoming and continuing students on fulfilling degree requirements and building coursework that supports their future goals. Maintained student records.
- Responsible for articulating transfer credits, evaluating applications for secondary bachelor degrees, and maintaining student records.
- Prepared the class schedule for USU's main campus Biology and Public Health Courses.

Instructor, Microbiology, Utah State University
2014 – 2015 Academic Year

- Instructor for BIOL 3300, General Microbiology with 80 students in Fall and 120 students in Spring semester.
- Developed all course materials with full latitude, including lectures, study guides, quizzes and exams.
- Supervised graduate teaching assistants in charge of the laboratory portion of the course.

- Initiated, supervised, and mentored undergraduate teaching assistants with great results in Spring semester.
- Supervised undergraduate technicians in charge of media preparation and disposal.

Lab Supervisor, Stable Isotope Lab, Utah State University
Jan. – Dec. 2015

- Hired, trained, and supervised technicians working in the laboratory.
- Responsible for ordering supplies and maintaining productivity.
- Advised customers on common soil sampling procedures and sample preparation.
- Work as the senior technician running Lachat QuickChem 8500 flow injection analyzer and Europa 20/20 continuous flow isotope ratio mass spectrometer, included instrument maintenance and troubleshooting.

Research Assistant, Stark Lab, Utah State University
Aug. 2011 – June 2014

- Developed and executed project for Master's thesis researching nitrogen cycling in the rhizosphere of *Bromus tectorum* (cheatgrass) and *Agropyron cristatum* (crested wheatgrass).
- Ran all samples for this research using Lachat QuickChem 8500 flow injection analyzer and Europa 20/20 continuous flow mass spectrometer.

Teaching Assistant, Microbiology, Utah State University
4 semesters (Fall 2012 – Spring 2014)

- Instructed two sections of General Microbiology lab per week consisting of ~30 students each.
- Taught aseptic technique, staining, physiological tests, and other crucial microbiology lab skills.
- Graded all lab related material, maintained equipment, prepared cultures and exam materials.

Teaching Assistant, Human Physiology, Utah State University
1 semester (Spring 2012)

- Instructed four sections of Human Physiology lab per week consisting of ~20 students each.
- Taught introductory lab skills, explained key concepts, and led students through laboratory exercises.
- Graded all laboratory related material, maintained equipment, and prepared exam materials for student use.

Biological Science Technician, Dinosaur National Monument, Dinosaur, CO, USA
Seasonal contracts for the summer of 2011 and 2010

- Trekked in the backcountry to Peregrine falcon (*Falco peregrinus*) territories to monitor breeding activity.
- Navigated by topographic map, GPS, and landmarks to remote areas, often alone on roads in poor conditions.
- Worked as part of the monument's Early Detection Rapid Response invasive plant management team.
- Collaborated with biocontrol monitor to evaluate the success of the tamarisk beetle (*Diorhabda carinulata*).
- Participated in a survey of rare and threatened orchid, Ute ladies' tresses (*Spiranthes diluvialis*).
- Often worked in teams of coworkers and volunteers ranging in size from two to fifteen (age range 13 to 70).

Field Technician, University of Nevada (Reno), Eureka County, NV
Aug. – Oct. 2009

- Monitored greater sage-grouse (*Centrocercus urophasianus*) using radio telemetry while living in a fieldcamp.
- Performed weekly walk-ins on a subset of hens to collect vegetation data documenting habitat use.
- This job required completely independent work in a remote and rugged area.
- A very similar position was held May to Aug. 2009, working for Utah State University in south central Utah.

Research Intern, Lyons Lab, Trinity University, San Antonio, TX
Summer 2008-Spring 2009

- Executed an individual research project regarding the impact of King Ranch bluestem (*Bothriochloa ischaemum*) on ant assemblages. Coordinated with a team during peak field season, processed pitfall trap samples, analyzed data, and presented results.

Scientific Merits

Publications

Underline indicates an undergraduate co-author.

- Gogesch, S. & K. A. Morris, *in prep* "N addition reduces C allocation by plants to arbuscular mycorrhizae". target journal TBD.
- Morris, K. A., P. Saetre, U. Norton, J. M. Stark, *in prep* "Soil moisture and plant community effects on nitrogen cycling in a semi-arid ecosystem". target journal: Plant and Soil.
- Morris, K. A., A. Richter, K. Küsel, M. Migliavacca, M. Schrumpf, *in prep* "Contrasting responses of microbial CUE to nitrogen and phosphorus addition in soils from two distinct habitats within one ecosystem". target journal: Soil Biology and Biochemistry.
- Nair, R. K. F., K. A. Morris, M. Migliavacca, G. Moreno, M. Schrumpf, *submitted* "Mediterranean savanna herbaceous roots respond to N:P imbalance". Plant and Soil. ID: PLSO-D-19-02012.
- Wutzler, T., O. Perez-Priego, K. A. Morris, T. El-Madany, M. Migliavacca, *in reivew*. "Soil CO_2 efflux errors are log-normally distributed - Implications and guidance". Geoscientific Instrumentation, Methods, and Data Systems. submission ID: gi-2019-10.
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- Nair, R. K. F., K. A. Morris, M. Hertel, Y. Lou, G. Moreno, M. Reichstein, M. Schrumpf, M. Migliavacca, 2019. "N:P stoichiometry and habitat effects on Mediterranean savanna seasonal root dynamics". Biogeosciences: 16, 1883-1901.
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- Durso, A. M. & K. A. Morris. 2015. *Agkistrodon contortrix* (Copperhead) diet. Natural History Note. Herpetological Review: 46(4) 636.

Select Conference Presentations

- Morris, K. A., K. Küsel, M. Schrumpf, M. Migliavacca, A. Richter, "Microbial community and soil properties modify microbial carbon use efficiency response to N and P addition" Soil Ecology Society 2019. Toledo, OH, USA (oral)
- Morris, K. A., T. Wutzler, M. Poehlmann, R. Nair, M. Schrumpf, M. Migliavacca, "Interaction of vegetative cover and N addition on soil CO_2 efflux in an oak savanna ecosystem" EGU 2017. Vienna, Austria (poster)
- Morris, K. A., R. Nair, M. Schrumpf, M. Migliavacca, "Soil Respiration and Partitioning in an Oak-Savannah with a History of Fertilizaiton" AGU 2017. New Orleans, LA, USA (poster)
- Morris, K. A., "Root exudates and senescence contribute to nitrogen cycling in the rhizosphere of cheatgrass and crested wheatgrass" ESA 2015. Baltimore, Maryland, USA (oral)

Awards and Achievements

- "Comparing Rhizosphere Nitrogen Cycling in *Bromus tectorum* and *Agropyron cristatum*", 2013, Ecology Center Research Support Award ($2,500)
- National Park Service STAR Award, Special Thanks for Achieving Results, 2011, awarded for exceptional work as a biological science technician at Dinosaur National Monument.
- Jacob Ulrich Scholarship, 2008, a scholarship awarded to rising seniors at Trinity University who show the interest and potential to pursue graduate studies in the biological sciences.

Guest Lectures

- *Biogeochemistry: Linking C to N & P*, Lower Trinity River Basin Chapter of Texas Master Naturalists, Aug. 2018
- *Intro to Biogeochemistry: C Cycling*, LTRB Chapter of Texas Master Naturalists, Dec. 2017
- *Microbes and You*, Biology and the Citizen (BIOL 1010), Utah State University, June 2015
- *Protists*, General Microbiology (BIOL 3300), Utah State University, Spring 2014
- *Viruses*, General Microbiology (BIOL 3300), Utah State University, Fall 2013

Public Outreach and Service

* denotes an elected position

- Work Council member 2018 – 2019*
- MaxNet External PhD representative 2016 – 2018*
- Long Night of Science 2017 & 2019, open-house event held city wide at scientific institutions
- USU Science Unwrapped (2-4 times per semester while living in Logan)
- Bridgerland Audubon Society fieldtrip co-leader (2-3 times per year, 2013-2015)

Special Skills

- Spoken German at B1 level, written level A2
- Bird identification by sight and sound for US Southern and Intermountain species as well as central Europe
- Flora, familiar with invasive species of the Intermountain West and Southeastern US

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